Sensory, chemical and consumer analysis of *Brettanomyces* spoilage in South African wines

Janita J. Botha

Thesis presented in partial fulfilment of the requirements for the degree of

Master of Science in Food Science

Stellenbosch University

Supervisors

M. Muller, Department of Food Science, Stellenbosch University

Dr A. G. J. Tredoux, Institute of Wine Biotechnology, Stellenbosch University

Dr W. J. du Toit, Department of Viticulture and Oenology, Stellenbosch University

Dr A. J. de Villiers, Department of Chemistry and Polymer Science, Stellenbosch University

March 2010

Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the owner of the copyright thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Date: 23/02/2010

Summary

This study focussed on the sensory effects of the main volatile compounds produced by *Brettanomyces* yeast causing spoilage in wine. This research firstly aimed to determine the detection thresholds of eight Brett-related spoilage compounds in wine. The second aim was to determine the sensory effect of the four most important Brett-related compounds when present individually in wine. The third aim was to determine the sensory effects of these four compounds when present in wine in a range of combinations, and to further investigate their effect on consumer liking. Finally, this project aimed to investigate the incidence of these compounds in a small range of South African wines.

The sensory detection thresholds of 4-ethylphenol, 4-ethylguaiacol, 4-ethylcatechol, 4-vinylphenol, 4-vinylguaiacol, isovaleric acid, isobutyric acid and acetic acid were determined. Apart from 4-ethylcatechol, these values generally agreed well with recent literature where values determined in wine are available. However, the discrepancies highlighted the importance of the effect of the medium (wine) when determining sensory detection thresholds. The use of the median as alternative calculation method was also investigated, and it was found that this method gives more insightful results than the standard American Society of Testing Materials (ASTM E679-04) method.

Four compounds, namely 4-ethylphenol, 4-ethylguaiacol, 4-ethylcatechol and isovaleric acid were profiled individually in wine using a trained sensory panel. It was found that all four compounds caused a suppression of the natural berry-like character in the wine, which induced a sick-sweet character. 4-ethylphenol contributed Elastoplast™ and leather aromas in the wine, both of which are commonly associated with *Brettanomyces* taint. 4-ethylguaiacol added a medicinal aroma to the wine, and 4-ethylcatechol and isovaleric acid were responsible for savoury and pungent aromas, respectively.

4-ethylphenol, 4-ethylguaiacol, 4-ethylcatechol and isovaleric acid were also profiled in combination according to the central composite design. Several univariate and multivariate methods were applied to the dataset obtained. PARAFAC, a multiway method not widely utilized regarding sensory data, was applied to the data, the results of which were complementary to those obtained during univariate and multivariate analyses. It was found that there is a great deal of interaction between the four compounds profiled in terms of sensory effects. The most notable was the Elastoplast™ attribute, the intensity of which was affected by all four compounds. The pungent attribute was also affected by the 4-ethylphenol concentration.

Consumer analysis revealed that some of the samples spiked with *Brettanomyces*-spoilage compounds were preferred to the unspiked (control sample). However, no further relationship could be found between consumer liking and either chemical composition or sensory profile. It is therefore speculated that consumer liking of *Brettanomyces* infected wine is driven by more complex sensory or socio-demographic factors.

Finally, the concentration of 4-ethylphenol, 4-ethylguaiacol, 4-ethylcatechol, 4-vinylphenol, 4-vinylguaiacol, isovaleric acid, isobutyric acid and acetic acid was determined in a small set of South African wines, selected to contain a high proportion of wines spoiled by *Brettanomyces*. Significant correlations were found between 4-ethylphenol and 4-ethylguaiacol, as well as 4-ethylphenol and isovaleric acid. However, no correlation could be found between 4-ethylphenol and 4-ethylcatechol. It is speculated that this lack of relationship is due to the different precursor profiles present in the analysed wines. This study paved the way for future investigations on the sensory effects of *Brettanomyces* spoilage in Pinotage red wine.

Opsomming

Hierdie studie het gefokus op die sensoriese invloed van die belangrikste vlugtige komponente wat deur die *Brettanomyces* gis geproduseer word en bederf veroorsaak in wyn. Eerstens is gefokus op die bepaling van die deteksiedrempelwaardes van agt Brett-verwante bederwende komponente. Die tweede doelwit was om die sensoriese invloed van vier van die mees belangrike Brett-komponente te bepaal wanneer hulle individueel in wyn voorkom. Die derde doelwit was om die sensoriese invloed van hierdie vier komponente te bepaal wanneer hulle in verskillende kombinasies in wyn voorkom, asook die effek daarvan op verbruikervoorkeur. Laastens is gepoog om die voorkoms van hierdie komponente in ‘n klein seleksie van Suid-Afrikaanse wyne te bepaal.

Die sensoriese deteksiedrempelwaardes vir 4-etielfenol, 4-etielguaiacol, 4-etielcatechol, 4-vinielfenol, 4-vinielguaiacol, isovaleraatsuur, isobuteraatsuur en asynsuur is bepaal. Met die uitsondering van 4-etielcatechol het die waardes oor die algemeen goed ooreengestem met waardes wat onlangs in die wetenskaplike literatuur gepubliseer is. Die uitsonderings het egter die belangrikheid van die medium (wyn) gedurende die bepaling van sensoriese deteksiedrempelwaardes uitgelig. Die gebruik van die mediaan as ‘n alternatiewe berekeningsmetode is ook ondersoek en daar is gevind dat hierdie metode meer insiggewende resultate lewer as die standaard American Society of Testing Materials (ASTM E679-04) metode.

Vier komponente naamlik 4-etielfenol, 4-etielguaiacol, 4-etielcatechol en isovaleraatsuur is individueel in wyn geprofileer met behulp van ‘n opgeleide sensoriese paneel. Daar is gevind dat al vier die komponente die natuurlike bessiekarakter in die wyn onderdruk terwyl dit aanleiding gee tot ‘n onnatuurlike soet karakter. 4-etielfenol is gekenmerk aan Elastoplast™ en leeragtige aromas in die wyn en beide van hulle word algemeen geassosieer met *Brettanomyces* bederf. 4-etielguaiacol het ‘n medisinale aroma tot die wyn toegevoeg en 4-etielcatechol en isovaleraatsuur het respektiewelik souterige (“savoury”) en sterk (“pungent”) aromas tot gevolg gehad.

4-etielfenol, 4-etielguaiacol, 4-etielcatechol en isovaleraatsuur is ook in verskeie kombinasies geprofileer volgens die sentrale saamgestelde ontwerp (“central composite design”). Verskeie enkelveranderlike en meerveranderlike statistiese analisemetodes is ook op die datastel uitgevoer. PARAFAC, ‘n meerrigtingsmetode wat nie normaalweg vir sensoriese analise data gebruik word nie, is ook uitgevoer op die data en die resultate was komplimentêr tot die van die enkelveranderlike en meerveranderlike analisemetodes. Daar is gevind dat, met betrekking tot

sensoriese effekte, daar noemenswaardige interaksie tussen die vier komponente plaasvind. Die mees opmerklike hiervan was die Elastoplast™ aroma, waarvan die intensiteit deur al vier die ander komponente geaffekteer is. Verder is die sterk ("pungent") aroma beïnvloed deur die 4-etielfenol konsentrasie.

Verbruikersvoorkeur-analise het aangedui dat sommige van die monsters waarby *Brettanomyces* bederwende komponente gevoeg is, verkies word bó die kontrole-wyn. Daar kon egter geen verdere verband gevind word tussen die verbruiker se voorkeur en, nog die chemise komposisie of sensoriese profiele, van die wyn nie. Daar kan dus gespekuleer word dat verbruiker voorkeur van *Brettanomyces* bederfde wyn gedryf word deur meer komplekse en sosio-demografiese faktore.

Laastens is die konsentrasies van 4-etielfenol, 4-etielguaiacol, 4-etielcatechol, 4-vinielfenol, 4-vinielguaiacol, isovaleraatsuur, isobuteraatsuur en asynsuur in 'n seleksie van Suid-Afrikaanse wyne bepaal. Dié wyne is spesifiek so gekies sodat 'n aansienlike aantal van hulle met *Brettanomyces* bederf was. Betekenisvolle korrelasies is gevind tussen 4-etielfenol and 4-etielguaiacol, sowel as 4-etielfenol en isovaleraatsuur. Daar is egter geen korrelasie tussen 4-etielfenol and 4-etielcatechol gevind nie. Daar word vermoed dat hierdie gebrek aan korrelasie te wyte is aan die voorloperkomponent profiele teenwoordig in die wyne. Hierdie studie het die weg gebaan vir verdere ondersoeke na die sensoriese effekte van *Brettanomyces* bederf in Pinotage rooi wyn.

Acknowledgements

Ms M. Muller for her input and continuous encouragement throughout the project, for always going the extra mile, and inspiring me to the same

Dr A. G. J. Tredoux for his input and support, as well as for persevering with the problem of 4-ethylcatechol analysis

Dr W. J. du Toit for his input, support and patience.

Dr A. J. de Villiers for his input, encouragement and being an exceptional editor.

Adriaan Oelofse and **Jan Bester** for their technical input and general enthusiasm for the project.

Frikkie Calitz and **Prof van Aarde** for their statistical input.

Thomas Skov, for his help with PARAFAC and his input into my manuscript.

Sensory Panel, without which there would be no thesis.

Department of Food Science, SU for use of facilities and hardworking personnel. Especially **Shantelle** for her vast amount of help in the sensory lab.

IWBT & Viticulture and Oenology, SU for use of facilities and personnel.

Distell for sponsorship of wine.

NRF for bursary.

My family, for support, prayers, laughter and repeatedly telling me that I DARE NOT quit! Especially my parents, **Johan** and **Benita**, for awakening the interests that led to this thesis, and always encouraging me to pursue excellence. **Mareli**, for being sister, friend and sometimes parent.

My friends (especially **Anomien**), for listening to me vent, and making me forget why I needed to vent in the first place!

My Heavenly Father, for blessing in terms of abilities and opportunities. I am constantly in awe of the Master plan.

Notes

The language and style used in this thesis are in accordance with the requirements of the scientific journal, International Journal of Food Science and Technology.

This thesis represents a compilation of manuscripts where each chapter is an individual entity and therefore some repetition between chapters may occur.

Table of contents

Chapter 1: Introduction

1 *BRETTANOMYCES*: THE CURRENT SITUATION

Wine can be spoiled by a number of organisms, including lactic acid bacteria, acetic acid bacteria and yeasts (Du Toit & Pretorius, 2000), as well as tainted from outside sources such as cork (Prescott *et al*., 2005). Wine spoilage generally results in a decrease in the quality of wine, and, if it is not detected before distribution, disappointment on the part of the wine consumer. However, consumer disappointment is directly related to the sensory effect of these wine taints, and not necessarily to the levels of spoilage compounds or spoilage organisms found in a wine. This leaves the wine industry with a particular problem, as the latter two are relatively easy to measure, but are not necessarily directly related to the sensory effect – which is the primary cause of consumer disappointment. Even one instance of disappointment can be enough to damage the brand of a wine to prevent a consumer from purchasing wines from the same vineyard, wine region or country of origin. For this reason, it is of utmost importance to not only define wine spoilage in terms of chemical and microbiological parameters, but also in terms of sensory and hedonic (consumer enjoyment) parameters (Charters & Pettigrew, 2007).

Brettanomyces is a wine spoilage organism that is related to several wine faults, most notably the fault originally known as phenolic off-flavour (Chatonnet *et al*., 1992) or Brett character[1]. This flavour can be described as horsey, leathery, medicinal, band-aid™, smoky or savoury (Chatonnet *et al.*, 1992; Licker *et al*., 1999; Wirz *et al*., 2004; Norris, 2004; Saurez *et al*., 2007; Romano *et al*., 2009). Although *Brettanomyces* is considered a spoilage organism and causes an objectionable flavour in red wine when its spoilage compounds are present in high levels, low levels of Brett character is sometimes considered to add complexity to a wine. While the physiological characteristics of the yeast are generally well explored, there has recently been a renewed interest in it, especially in terms of molecular detection methods for the yeast (Campolongo *et al*., 2009; Oelofse *et al*., 2009), chemical detection methods for its spoilage compounds (Boutou & Chatonnet, 2007; Carillo & Tena 2007; Cyncar *et al*., 2007; Fariña *et al*., 2007; Larcher *et al*., 2007; Pizarro *et al*., 2007; Larcher *et al*., 2008; Hisomoto *et al*., 2009) and its sensory effects (Curtin *et al*., 2008; Cliff & King, 2009; Romano *et al*., 2009). This can partially be ascribed to the fact that more sophisticated methodologies have been developed, which have made these studies possible. Furthermore, there has been an increased awareness of sensory science, the appropriate methodologies for performing sensory tests and the possibilities of what can be achieved with sensory science (Tuorila & Monteleone, 2009).

[1] The terms Brett character, “Brettyness” or simply “Brett” are used in literature to refer to the sensory effect of wine spoiled by *Brettanomyces.* As far as possible, the term Brett character will be used throughout.

The two main spoilage compounds traditionally associated with *Brettanomyces* are 4-ethylphenol and 4-ethylguaiacol. The aroma of 4-ethylphenol is associated with leather/band-aid™ while 4-ethylguaiacol has a medicinal/spicy smell associated with it (Saurez *et al*., 2007). These compounds are generally considered indicator compounds for spoilage by *Brettanomyces*. The combined rejection threshold of a total combined concentration of these two compounds of 426 µg/L is generally used as diagnostic criterion for wine potentially spoiled with *Brettanomyces* (Chatonnet *et al*., 1992). However, poor qualitative correlations have been found between the presence of these compounds and their sensory effects (Romano *et al*., 2009). This may be ascribed to sensory interactions between these compounds, some of the other compounds associated with *Brettanomyces* spoilage, as well as other compounds originating from the grapes, alcoholic fermentation and ageing.

Two other compounds of particular interest are isovaleric acid and 4-ethylcatechol. Isovaleric acid is formed by the metabolism of L-leucine (Harwood & Canale-Parola, 1981), and there has been much debate on its sensory effect on wines spoiled by *Brettanomyces* (Licker *et al*., 1999; Fugelsang & Zoecklein, 2003; Romano *et al*., 2008; Romano *et al*., 2009). 4-ethylcatechol is formed in an analogous manner to 4-ethylphenol and 4-ethylguaiacol, but has only recently been linked to *Brettanomyces* spoilage due to the fact that 4-ethylcatechol cannot be detected by the same chemical analysis methods as 4-ethylphenol and 4-ethylguaiacol due to its lower volatility (Hesford & Schneider, 2004; Hesford *et al*., 2004; Carillo & Tena, 2007). The sensory effects of 4-ethylcatechol in wine are still poorly understood (Curtin *et al.,* 2008; Larcher *et al.,* 2008). In the South African wine industry, chemical diagnosis of *Brettanomyces* spoilage is generally limited to testing for elevated 4-ethylphenol and 4-ethylguaiacol concentrations. However, Pinotage a uniquely South African wine cultivar, contains significantly higher levels of the precursors of 4-ethylcatechol (De Viliers *et al*., 2005). This makes investigation into the sensory and chemical effects of this yeast in Pinotage wine of utmost importance

Limited studies have been performed on the effect of *Brettanomyces*-related spoilage compounds and the acceptability of wines (Etiévant *et al*., 1989; Chatonnet *et al*., 1992), although recent studies by the Australian Wine Research Institute (AWRI) have found that Australian consumers find wines tainted with *Brettanomyces* less acceptable than untainted wines (Lattey *et al*., 2007; Curtin *et al*., 2008).

The field of sensometrics investigates relationships between chemical profiles, sensory descriptors and hedonics. When hedonics is mapped against other wine characteristics, the statistical technique is known as Preference Mapping. This technique has been applied to wines (Frøst & Noble, 2002), but is not generally used to investigate wine taints. However, the fact that this technique can use a bipolar scale such as the nine-point hedonic scale, and can therefore be used to measure both positive and negative hedonic responses, makes this technique ideal

for investigating the effect of *Brettanomyces* spoilage compounds and their effects on the consumer preference of wines.

The stigma that is attached to *Brettanomyces* by the wine industry is a hurdle faced by researchers, as winemakers are embarrassed to admit that they might have a problem regarding Brett and therefore reluctant to co-operate with research. In spite of the widespread denial by winemakers that they have a Brett problem, Australian winemaker Brian Crosser commented that Brett character was prevalent amongst wines tasted at a recent prestigious South African wine show (Eedes, 2009). The anecdotal prevalence of this defect makes investigation into *Brettanomyces* spoilage in the South African context relevant and absolutely necessary.

.

2 RESEARCH AIMS

The overall aim of this study was to systematically investigate the sensory effects of *Brettanomyces* spoilage compounds in South African wine. This was done by combining sensory profiling with chemical and consumer analysis. The specific aims of the project can be summarised as follows:

i) To determine the sensory detection thresholds of eight compounds originating from *Brettanomyces* infection in Pinotage wine (Chapter 3).

ii) To determine the sensory effects of 4-ethylphenol, 4-ethylguaiacol, 4-ethylcatechol and isovaleric acid on the sensory profile of Pinotage red wine spiked with these compounds (Chapter 4).

iii) To determine the sensory interactions between the above-mentioned four compounds on the sensory profile of Pinotage red wine spiked with these compounds (Chapter 5).

iv) To determine the effect of the above-mentioned four compounds on consumer acceptance of Pinotage red wine spiked with these compounds (Chapter 5).

v) To investigate the prevalence of *Brettanomyces*-related spoilage compounds in South African red wines (Chapter 6).

3 REFERENCES

Boutou, S. & Chatonnet, P. (2007) Rapid headspace solid-phase microextraction/gas chromatographic/mass spectrometric assay for the quantitative determination of some of the main odorants causing off-flavours in wine. *Journal of Chromatography A*. **1141**, 1 - 9 .

Campolongo, S., Rantsiou, K., Giordano, M., Gerbi, V. & Cocolin, L. (2009) *Brettanomyces bruxellensis* incidence and diversity in Italian wines as determined by molecular methods (abstract). *American Journal of Enology and Viticulture*. **60**, 398.

Carrillo, J. D. & Tena, M. T. (2007) Determination of ethylphenols in wine by in situ derivitisation and headspace solid-phase microextraction-gas chromatography-mass spectrometry. *Analytical and Bioanalytical Chemistry*. **387**, 2547 - 2558.

Charters, S. & Pettigrew, S. (2007) The dimensions of wine quality. *Food Quality and Preference.* **15**, 997 - 1009.

Chatonnet, P., Dubourdieu, D., Boidron, J. & Pons, M. (1992) The origin of ethylphenols in wines. *Journal of the Science of Food and Agriculture*. **60**, 165 - 175.

Cliff, M. A. & King, M. C. (2009) Influence of serving temperature and wine type on perception of ethyl acetate and 4-ethyl phenol in wine. *Journal of Wine Research*. **20**, 45 - 52.

Curtin, C. Bramley, B. Cowey, G. Holdstock, M. Kennedy, E. Lattey, K. Coulter, A. Henschke, P. Francis, L. Godden, P. Sensory perceptions of 'Brett' and relationship to consumer preference. Blair, R.J.; Williams, P.J.; Pretorius, I.S. Proceedings of the thirteenth Australian wine industry technical conference, 29 July-2 August 2007, Adelaide, SA. : 207-211; 2008.

Cyncar, W., Cozzolino, D., Dambergs, B., Janik, L. & Gishen, M. (2007) Feasibility study on the use of a head space mass spectrometry electronic nose (MS e_nose) to monitor red wine spoilage induced by *Brettanomyces* yeast. *Sensors and Actuators*. **124**, 167 - 171.

De Villiers, A., Majek, P., Lynen, F., Crouch, A., Lauer, H. & Sandra, P. (2005) Classification of South African red and white wines according to grape variety based on the non-coloured phenolic content. *European Food Research and Technology*. **221**, 520 - 528.

Du Toit, M. & Pretorius, I. S. (2000) Microbial spoilage and preservation of wine: Using weapons from nature's own arsenal - a review. *South African Journal of Enology and Viticulture*. **21**, 74 -96.

Eedes, C (2009). Brett in evidence at Old Mutual Trophy Wine Show 2009 [WWW document]. http://www.winemag.co.za/article/hallo-darkness-my-old-friend-2009-06-24 29 October 2009.

Etiévant, P. X., Issanchou, S. N., Marie, S., Ducruet, V. & Flanzy, C. (1989) Sensory impact of volatile phenols on red wine aroma: influence of carbonic maceration time and storage. *Sciences des aliments*. **9**, 19 - 31.

Fariña, L., Boido, E., Carrau, F. & Dellacassa, E. (2007) Determination of volatile phenols in red wines by dispersive liquid-liquid microextraction and gas chromatography-mass spectrometry detection. *Journal of Chromatography A*. **1157**, 46 - 50.

Frøst, M. B. & Noble, A. C. (2002) Preliminary study on the effect of knowledge and sensory expertise on liking for red wines. *American Journal of Enology and Viticulture*. **53**, 275 - 284.

Fugelsang, K. C. & Zoecklein, B. W. (2003) Population dynamics and effects of *Brettanomyces bruxellensis* strains on Pinot noir (Vitis vinifera L.) wines. *American Journal of Enology and Viticulture*. **54**, 294 - 300.

Harwood C. S. & Canale-Parola, E. (1981) Adenosine-5'-Triphosphate-yielding pathways of branched-chain amino acid fermentation by a marine Spirochete. *Journal of Bacteriology*. **148**, 117 - 123.

Hesford, F. & Schneider, K. (2004) Discovery of a third ethylphenol contributing to *Brettanomyces* taint. *Obst- und Weinbau*. **140**, 11-13.

Hesford, F., Schneider, K., Porret, N. & Gafner, J. (2004) Identification and analysis of 4-ethyl catechol in wines tainted by *Brettanomyces* off-flavor (abstract). *American Journal of Enology and Viticulture*. **55**, 304A.

Hisomoto, M., Okuda, T., Nishimoto, S., Tani, K., Tachibana, M., Koizumi, H., Kiba, N. & Yokotsuka, K. (2009) Determination of 4-vinylcatechol in wine by HPLC-DAD coupled with flourescence detection (abstract). *American Journal of Enology and Viticulture*. **60**, 402.

Larcher, R., Nicolini, G., Bertoldi, D. & Nardin, T. (2008) Determinination of 4-ethylcatechol in wine by high-performance liquid chromatography-coulometric electrochemical array detection. *Analytica Chimica Acta*. **609**, 235 - 240.

Larcher, R., Nicolini, G., Puecher, C., Bertoldi, D., Moser, S. & Favaro, G. (2007) Determination of volatile phenols in wine using high-performance liquid chromatography with a coulometric array detector. *Analytica Chimica Acta*. **582**, 55 -60.

Lattey, K. A., Bramley, B. R., Francis, I. L., Herderich, M. J. & Pretorius, S. (2007) Wine quality and consumer preferences: understanding consumer needs. *Australian and New Zealand Wine industry Journal.* **22**, 31 - 39.

Licker, J. L, Acree, T. E. & Henick-Kling, T. (1999) What is "Brett" (*Brettanomyces*) flavour? A preliminary investigation. In: Chemistry of Wine Flavour. ACS Symposium Series (edited by A. L. Waterhouse & S. E. Ebeler), Pp 96 - 115. Washington DC: American Chemical Society.

Norris, L. (2004) Unraveling the mystery of *Brettanomyces* flavor (abstract). *American Journal of Enology and Viticulture*. **55**, 304A.

Oelofse, A., Lonvaud-Funel, A. & du Toit, M. (2009) Molecular identification of *Brettanomyces bruxellensis* strains isolated from red wines and volatile phenol production. *Food Microbiology*. **26**, 377 - 385.

Pizarro, C., Pérez-del-Notario, N. & González-Sáiz, J. M. (2007) Determination of Brett character responsible compounds in wines by using multiple headspace solid-phase microextraction. *Journal of Chromatography A*. **1143**, 176 - 181.

Prescott, J., Norris, L., Kunst, M. & Kim, S. (2005) Estimating a "consumer rejection threshold" for cork taint in white wine. *Food Quality and Preference*. **16**, 345 - 349.

Romano, A., Perello, M. C., de Revel, G. & Lonvaud-Funel, A. (2008) Growth and volatlie compound production by *Brettanomyces/Dekkera bruxellensis* in red wine. *Journal of Applied Microbiology*. **104**, 1577 - 1585.

Romano, A., Perello, M. C., Lonvaud-Funel, A., Silcard, G. & de Revel, G. (2009) Sensory and analytical re-evaluation of “Brett character”. *Food Chemistry*. **114**, 15 - 19.

Saurez, R., Saurez-Lepe, J. A., Morata, A. & Calderon, F. (2007) The production of ethylphenols in wine by yeasts of the genera *Brettanomyces* and *Dekkera*: A review. *Food Chemistry*. **102**, 10-21.

Tuorila, H. & Monteleone, E. (2009) Sensory food science in the changing society: Opportunities, needs, and challenges. *Trends in Food Science & Technology*. **20**, 64 - 62.

Wirz, D. O., Heymann, H. & Bisson, L. F. (2004) Descriptive analysis of *Brettanomyces*-infected Cabernet Sauvignon wines. *American Journal of Enology and Viticulture*. **55**, 303A.

Chapter 2: Literature Review: *Brettanomyces* in red wine

1 INTRODUCTION

Wine is aged in order to improve the olfactory, gustatory and visual quality of the product. The practice of aging wines also allows winemakers to control the release of their products into the marketplace. Unfortunately, wine spoilage can take place during aging in wooden barrels due to the presence of undesirable yeasts and other organisms remaining in the pores of wooden barrels after cleansing and sterilisation. The risk of spoilage is greater when the aging period of a particular wine is extended. Production trends in the wine industry also have an influence on this type of spoilage (Suarez *et al.*, 2007).

Current winemaking trends seem to encourage subjecting wine to limited stabilisation processes. In other words, wines are not subjected to clarification or to physical treatments such as filtration. The philosophy behind this trend is to maintain high concentrations of aromatic compounds, pigments and colloids in the wine, in order to allow the product to reach its full potential. Filtering under certain conditions, for example, has been shown to have a negative effect on the mouthfeel, body and aroma of a wine (Arriagada-Carrazana *et al.,* 2005). The use of sulphur dioxide – a preservative that is commonly used in winemaking, is also discouraged. Another, relatively new trend, is to allow red wine grapes to ripen to a stage of almost over-ripeness, which increases the polyphenol content of wines, and therefore adds to its character. This results in a wine with a lower acidity. The higher degree of ripeness (and therefore sugar content) ultimately leads to a higher alcohol concentration, which causes primary fermentation and malolactic fermentation to take longer to complete (Kelly, 2003). Unfortunately, the combined effect of these trends is that the growth of wine spoilage organisms – like *Brettanomyces* – is more prevalent than ever before.

Brett character is a wine defect associated with an unpleasant aroma reminiscent of medicine, leather, horse-sweat or band-aid™ (Elastoplast™) which most often occurs in red wine (Du Toit *et al*., 2005). It is caused by spoilage by the yeast *Brettanomyces*, and its sporulating form, *Dekkera* (Loureiro & Malfeito-Ferreira, 2003). The cause of this defect was first thought to occur during malolactic fermentation, as certain lactic acid bacteria can produce ethylphenols and the latter are commonly associated with this defect. However, it was found that these species are not able to produce ethylphenols at the elevated levels associated with Brett character under typical winemaking conditions (Chatonnet *et al*., 1995; Suarez *et al*., 2007). The fact that the sensory defect associated with *Brettanomyces* more generally occurs in red wine, is possibly due to the inability of *Brettanomyces* to survive in white wine (Barata *et al*., 2008), as well as the combined effects of higher pH and increased levels of polyphenols in red wines. Spoilage of this type has also been found to be more common during the summer months (Chatonnet *et al*., 1992). Although the defect is more commonly found in wines aged in barrels, in particularly old barrels, it has also been found in wines not aged in barrels (Chatonnet *et al*., 1992; Rodriguez *et al*., 2001).

The spoilage of wine by *Brettanomyces*/*Dekkera* is not a novel occurrence, as the South African wine industry has been dealing with this problem for the past 40 years. It is also historically associated with some of the wines made in the Bordeaux and Burgundy regions of France. A survey done in New Zealand in the early 1970's showed that *Brettanomyces* was widespread in this industry, although it could not be detected in winery interiors or on winery equipment with the methods then available. No links could be found at that time between the presence of *Brettanomyces* and winemaking conditions (Wright & Parle, 1974). However, there appears to be a recent international rise in incidence of this defect, possibly due to winemaking trends and modern winemaking techniques which encourage the survival and growth of *Brettanomyces.*

2 INCIDENCE OF *BRETTANOMYCES* SPOILAGE

The wine industry is increasingly concerned about the presence of *Brettanomyces* in wines (Rayne & Eggers, 2007), and there have been several anecdotal reports of an increase in incidence of this defect (Kelly, 2003; Saurez *et al.*, 2007). However, a survey done of Australian red wines between 1996/1997 and 2002 showed a decrease in mean 4-ethylphenol concentration in wines from between 1000 and 1200 µg/L in 1996 to approximately 400 µg/L in 2002. This drop in 4-ethylphenol levels is ascribed to the initiative of the Australian Wine Research Institute (AWRI) to promote optimal SO_2 use in wines (Godden & Gishen, 2005). However, this average level is only slightly lower than the additive threshold, 426 µg/L, the total concentration at which Chatonnet *et al.* (1992) determined the combination of 4-ethylphenol and 4-ethylguaiacol to have a negative effect on the sensory quality of a Bordeaux-style red wine. This means that although the average level has dropped during the above-mentioned period, 4-ethylphenol concentrations may still be at levels at which their aroma contribution becomes objectionable in a large number of the wines tested.

It is difficult to accurately determine the "incidence" of *Brettanomyces* spoilage due to several factors. The first is the fact that *Brettanomyces* spoilage can be "measured" or diagnosed in different ways, and there are often poor correlations between the different methods. The most obvious way of measuring the incidence of *Brettanomyces* spoilage is by determining cell numbers. However, two complications arise here, namely the fact that cell numbers are not directly related to the levels of ethylphenols found in wines (Fugelsang & Zoecklein, 2003) and that *Brettanomyces* populations are difficult to measure accurately using traditional microbiological methods such as selective plating, due to the viable but non-culturable nature of this organism (Millet & Lonvuad-Funel, 2000). The levels of ethylphenols in wine have also been shown to be poorly correlated to the sensory effect that is known as "Brettyness" or phenolic taint (Romano *et al.*, 2008), which complicates the problem even further. Finally, *Brettanomyces* spoilage as such is still a debatable issue, as people differ in

their opinion on the perceived contribution of the metabolites produced by this yeast to wine quality. More recent studies have used combinations of culturing, molecular microbiology (PCR) and analytical chemistry techniques for the determination of incidence of *Brettanomyces* spoilage (Campolongo *et al*., 2009).

3 MICROBIOLOGICAL FACTORS CONTRIBUTING TO BRETT SPOILAGE

Several factors influence the presence of *Brettanomyces* in a wine environment. The degree and method of contamination, as well as nutritional factors and inhibitory agents all play a role.

3.1 *Brettanomyces* contamination

Wooden wine barrels are porous containers that are extremely difficult to clean and even more difficult to sterilise, and can provide an excellent environment for the survival and transfer of undesirable microorganisms such as *Brettanomyces* (Saurez *et al.,* 2007). However, used barrels are not the only source of contamination, as the sensory defect has been shown to occur in wines that have had no contact with barrels, as well as wines aged in new barrels (Chatonnet *et al.*, 1992; Rodriguez *et al.*, 2001). *Brettanomyces bruxellensis* has been found on grape skins (Renouf *et al.*, 2007; Renouf *et al.*, 2007a) and this is considered an important source of contamination. A correlation between the presence of *Brettanomyces* and *Botrytis* on grape skins has also been noted. This may be because excessive heat and moisture both favour *Brettanomyces* and *Botrytis*, rather than a direct interaction between the two species. Damaged grapes can enhance the development of *Brettanomyces* on berries, as nutrients previously trapped in the berries are liberated (Renouf *et al.,* 2007). The reintroduction of contaminated lees during aging can also introduce *Brettanomyces bruxellensis* into wine. *Brettanomyces* is also commonly found in vats, pumps and equipment that is difficult to sterilise (Suarez *et al.*, 2007).

3.2 Factors influencing *Brettanomyces* spp. growth

Brettanomyces possesses several competitive advantages over other microbial genera that can occur in wine. It can survive in the nutritionally poor environment in a wine following completion of malolactic fermentation, and can even use ethanol as sole carbon source (Uscanga *et al.*, 2000; Rodriguez *et al.,* 2001; Silva *et al.,* 2004). It is tolerant to slightly lower ethanol levels than *Saccharomyces cerevisea*: the upper limit of resistance is said to be at 14.5 - 15 % (Barata *et al.,* 2008).

Brettanomyces has been found to be the only surviving micro-organism in wine after bottling, due to its ability to survive in the anaerobic conditions (Renouf *et al.,* 2007). However, *Brettanomyces* can also grow in aerobic conditions, although its characteristics as an organism are slightly different than those found under anaerobic conditions.

Under fully aerobic conditions *Brettanomyces* multiplies more quickly and produces large volumes of acetic acid and small volumes of ethanol. Semi-aerobic conditions cause the production of less acetic acid. In aerobic conditions, *Brettanomyces* has been shown to display a loss of viability after 200 hours (Ciani & Ferrero, 1997).

The total cell numbers produced during anaerobic conditions are also higher than those produced during aerobic conditions (Ciani & Ferrero, 1997). Under anaerobic conditions, *Brettanomyces* can still ferment even though it is not multiplying. It also produces acetaldehyde under these conditions, which has the capacity to bind free SO_2 in wine, making conditions for *Brettanomyces* growth even more favourable (Ciani *et al.*, 2003).

Growth of *Brettanomyces bruxellensis* can be stimulated by the addition of ammonium sulphate or yeast extract to a medium (Uscanga *et al.*, 2000). Biotin and thiamine are both required for growth of this organism (Conterno *et al.*, 2006).

This organism also has the ability to resume growth after an apparent death phase should the conditions for growth become more favourable, having a viable but non-culturable state as result (Barata *et al.,* 2008). The “viable” numbers of *Brettanomyces* can be up to ten times as large as the culturable population, but these differences are negated as the organism resumes growth (Millet & Lonvaud-Funel, 2000).

The genus is quite sensitive to SO_2, from a level of 0.25 to 0.35 mg/L molecular SO_2 (Du Toit *et al.,* 2005). Barata *et al.* (2008) reported a slightly lower sensitivity to SO_2, and recommend adjusting the level of molecular SO_2 in wine to 1.0 mg/L before barrel ageing. The presence of oxygen also reduces the sensitivity of *Brettanomyces* to SO_2, but strain differences also play a role in SO_2 sensitivity (Du Toit *et al.*, 2005).

4 SENSORY CHARACTERISTICS OF *BRETTANOMYCES* SPOILAGE

Sensory descriptors for wine with Brett character include rancid, band-aid, soy, horsey, leather, tobacco and putrid (Wirz *et al.*, 2004). Brett character also masks inherent fruitiness in wines, as well as varietal character (Licker *et al.*, 1999; Fugelsang & Zoecklein, 2003; Farina *et al.*, 2007). The production of acetic acid by *Brettanomyces* increases the acidity (and therefore sour taste) of a wine. However, the perception of Brett character is also dependant on wine style and variety (Saurez *et al.*, 2007; Curtin *et al*., 2008), and fruity, low-tannin red wines do not tolerate a large amount of “Brettyness” (Norris, 2004).

5 CHEMICAL COMPOUNDS RESPONSIBLE FOR BRETT CHARACTER

Several different compounds have been linked to Brett character, of which the volatile phenols are most commonly associated with this spoilage defect. Other compounds, most notably isovaleric acid, have also been linked to this defect by different authors, resulting in some controversy. These compounds are summarised in Table 2.1 and Table 2.2 and will be discussed in the following sections. In these tables, the term "Brett compound" is used, which refers to a compound commonly accepted to be related to *Brettanomyces* spoilage.

Table 2.1. Volatile phenols and breakdown products linked with Brett character. The term "Brett compound" refers to a compound commonly accepted to be related to *Brettanomyces* spoilage.

Compound	Source	Odour	Reference	Status
4-ethylphenol (4-EP)	Conversion of 4-vinylphenol by vinylphenol reductase	Leather, Elastoplast™ or band-aid™	Chatonnet *et a.l*, 1992	Main spoilage compound
4-ethylguaiacol (4-EG)	Conversion of 4-vinylguaiacol by vinylphenol reductase	Medicinal	Chatonnet *et al*., 1992	Main spoilage compound
4-ethylcatechol (4-EC)	Conversion of 4-vinylcatechol by vinylphenol reductase	Horsey, smoky	Hesford *et al*., 2004	Recently accepted
4-vinylphenol (4-VP)	Conversion of ferulic acid by hydrocinnamate decarboxilase	Almond shell	Chatonnet *et al.*, 1992	Accepted as minor spoilage compound
4-vinylguaiacol (4-VG)	Conversion of *p*-coumaric acid by hydrocinnamate decarboxilase	Flowery, spicy	Chatonnet *et al*., 1992	Accepted as minor spoilage compound
4-vinylcatechol (4-VC)	Conversion of caffeic acid by hydrocinnamate decarboxilase	Phenolic, medicinal, smoky	Hisomoto *et al*., 2009	Not generally accepted as Brett compound
4-hydroxy-acetophenone	Breakdown product of 4-ethylphenol	Sweet, floral	Rayne & Eggers, 2007	Not considered Brett compound
4-acetovanilone	Breakdown product of 4-ethylguaiacol	Vanilla	Rayne & Eggers, 2007	Not considered Brett compound

5.1 Volatile phenols and sensory impact

Limited work has been done on the sensory effects of *Brettanomyces* spoilage, although some studies have attempted to link the sensory effects of *Brettanomyces* with chemical compounds. The existence of the volatile phenols (4-vinylphenol, 4-vinylguaiacol, 4-ethylphenol and 4-ethylguaiacol) in red wines has been known since the 1960's (Etiévant *et al.*, 1989; Chatonnet *et al.*, 1992), but their presence was originally thought to be of bacterial origin. A threshold at which 4-ethylphenol would contribute negatively to the wine character has been determined to

lie between 1200 and 2400 µg/L, with a level where a phenolic smell became apparent set at 4300 µg/L. The influence of maceration type and aging time on 4-ethylphenol concentration was also apparent (Eteiévant *et al.*, 1989).

Table 2.2. Compounds that may be associated with Brett character. The term "Brett compound" refers to a compound commonly accepted to be related to *Brettanomyces* spoilage.

Compound	Source	Descriptor	Linked by	Status
Isovaleric acid	Breakdown product of L-Leucine	Sweaty, rancid	Licker *et al*., 1999	Controversial, but generally accepted
Isobutyric acid	Breakdown product of L-leucine	Sweaty, rancid	Romano *et al*., 2009	Not considered Brett compound
2-phenyl ethanol	N/C[a]	Spicy	Licker *et al*., 1999	Not considered Brett compound
Ethyl decanoate	N/C	Plastic	Licker *et al*., 1999	Not considered Brett compound
cis-2-nonenal trans-2-nonenal	N/C	Burning tyres	Licker *et al*., 1999	Not considered Brett compound
Guaiacol	N/C	Plastic	Licker *et al*., 1999	Not considered Brett compound
4-propylguiacol	Extraction from oak wood	Spicy	Ferreira *et al*., 2006	Not considered Brett compound
Acetaldehyde	N/C	Sherry, nutty, bruised apple	Ciani *et al*., 2003	Not considered Brett compound
Ethyl acetate	N/C	Nail polish, fruity	Ciani *et al*., 2003	Not considered Brett compound

[a] Not confirmed

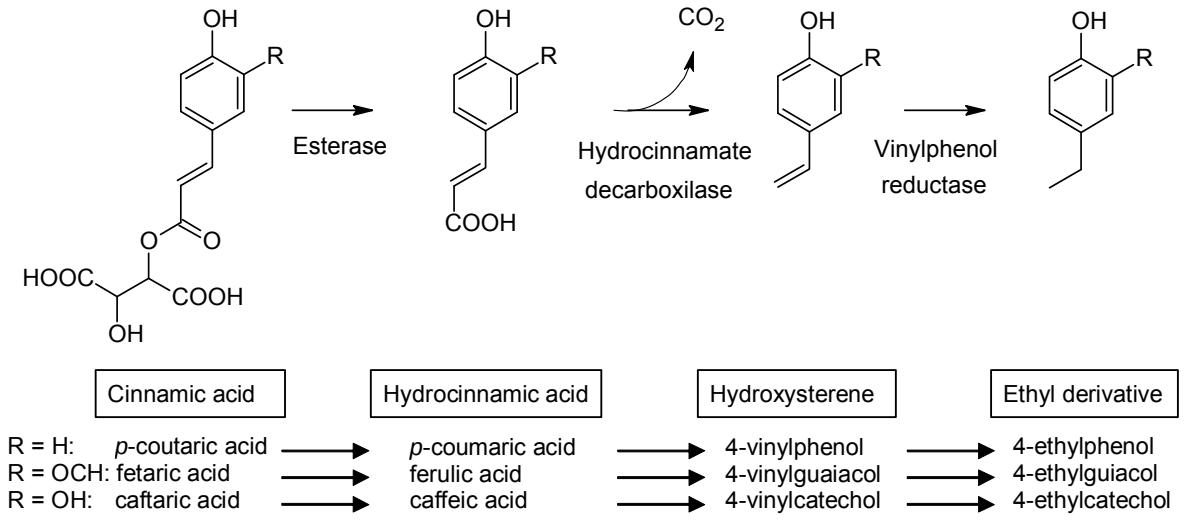


Figure 2.1. Formation of ethylphenols from cinnamic acid precursors (Oelofse *et al*., 2008).

Some of the first studies linking *Brettanomyces bruxellensis* to ethylphenols were performed by Chatonnet *et al.* (1992, 1993). These studies confirmed that the microbiological origin of ethylphenols in red wines is indeed the yeast *Brettanomyces,* and not lactic acid bacteria present during malolactic fermentation, or the yeast *Saccharomyces cerivisae,* as originally thought. *Saccharomyces cerivisae* (Chatonnet *et al.*, 1993), other yeast species and *Oenococcus oeni* can produce 4-vinylphenol and 4-vinylguaiacol from ferulic and *para*-coumaric acid, through the action of hydrocinnamate decarboxilase (Chatonnet *et al.*, 1995), but only *Lactobacillus plantarum* and *Dekkera/Brettanomyces* possess the enzyme vinylphenol reductase which converts the vinylphenols to their respective ethylphenols (Chatonnet *et al.*, 1995). The action of the two above-mentioned enzymes is shown in Figure 2.1. Only *Dekkera*/*Brettanomyces* can produce ethylphenols at the levels found in wines, and have a 50-60% conversion rate of the available substrate (Chatonnet *et al.*, 1992). The production of volatile phenols by other organisms is also inhibited by higher levels of polyphenols in wines. However, their production by *Dekkera/Brettanomyces* is not inhibited (Chatonnet *et al.*, 1993).

Two theories exist about the reason for the conversion of hydrocinnamic acids to volatile phenols. The first is that the yeast recovers energy from the decarboxylation/reduction reaction in the form of an electron gradient, which allows for adenosine-5'-triphospate (ATP) production. The second is that the yeast detoxifies its environment by this conversion. Phenolic acids have the capacity to deteriorate the plasmic membrane by destroying the phospholipid bi-layer. The degradation of phenolic acids may therefore inhibit their action on cell destruction (Renouf *et al.*, 2007).

Chatonnet *et al*. (1992) also explored the sensory impact of volatile phenols in red wines. The perception thresholds of 4-ethylphenol and 4-ethylguaiacol were determined using a method where the detection threshold was defined as the minimum concentration below which 50% of the large number of tasters (70) failed to detect a difference from the control. The determination of perception thresholds was done in a hydro-alcoholic model solution, as well as in water. In the model solution, thresholds were found to be 440 µg/L and 47 µg/L for 4-ethylphenol and 4-ethylguaiacol, respectively, whereas in water they were found to be 130 µg/L and 35 µg/L, respectively. They also analysed wines with "phenolic", "animal" and "stable" characteristics by Gas Chromatography–Olfactometry (GC-O) in order to determine which volatile substances were related to the olfactory faults. These authors concluded that 4-ethylguaiacol, 4-ethylphenol and 4-vinylphenol are associated with the defect. 4-ethylphenol gave the most intense "stable" characteristic; 4-ethylguaiacol had a spicy/phenolic smell, whereas 4-vinylphenol had the lowest intensity and had a medicinal smell associated with to it. The sensory interaction between 4-ethylguaiacol and 4-ethylphenol was also investigated, and indications of an interaction in terms of detection threshold and sensory impact were observed. However, this interaction was not clearly defined. It has also been shown that 4-vinylphenol

masks fruity nuances below its perception threshold, and that 4-vinylguaiacol adds flowery and spicy notes to the aroma of a wine (Chatonnet *et al*., 1992).

Another volatile phenol recently reported in Brettanomyces-infected wines is 4-ethylcatechol, which has a horsey flavour and is formed from caffeic acid in an analogous manner to 4-ethylphenol and 4-ethylguaiacol from p-coumaric and p-ferulic acid, respectively (Figure 2.1). The levels of 4-ethylcatechol produced were related to the grape variety (Hesford et al., 2004). This finding was confirmed by Larcher *et al.* (2008). This is most likely due to the different profiles of hydrocinnamic acid precursors present in the wines produced from different varieties. It is interesting to note that Pinotage, a uniquely South African cultivar, contains significantly higher levels of caffeic acid and its precursor, caftaric acid, than other wine cultivars (de Villiers et al., 2005; Rossouw & Marias, 2004), making this cultivar more susceptible to spoilage by 4-ethylcatechol. However, Larcher *et al.* (2008) postulates that 4-ethylcatechol probably does not have a large negative effect on the sensory profiles of wines. Regardless, this makes for an interesting topic for investigation.

The detection thresholds determined of 4-ethylcatechol has been determined by three authors to date, but differ drastically. The three values obtained were: 60 µg/L (Hesford & Schneider, 2004), 100 – 400 µg/L (Larcher *et al*., 2008) and 774 µg/L (Curtin *et al*., 2008). All these values fall below the maximum level of 1610 µg/L of 4-ethylcatechol that has been found in wine to date (Larcher *et al*., 2008), but these discrepancies warrants further investigation into this compound.

5.2 Volatile phenol breakdown products

The stability of ethylphenols in red wine influences their concentrations in wine, as well as the resulting sensory profile. This aspect is still poorly understood, although it is known that 4-ethylphenol can be broken down into 4-hydroxyacetophenone via the enzyme 4-ethylphenol methylenehydroxylase (4EPMH) (Figure 2.2). Another enzyme, p-cresol methylhydroxylase (PCMH), breaks down 4-ethylphenol to 4-hydroxyacetophenone and 4-vinylphenol. Similarly, the breakdown products of 4-ethylguaiacol are 4-vinylguaiacol and 4-acetovanillone (Figure 2.3). These breakdown products can influence the sensory profiles in Brettanomyces-infected wines, as they all have their own specific flavours; 4-hydroxyacetophenone has a sweet, floral aroma, 4-vinylphenol has an almond shell aroma, 4-vinylguaiacol a clove-curry aroma, and 4-acetovanillone a vanilla aroma (Rayne & Eggers, 2007).

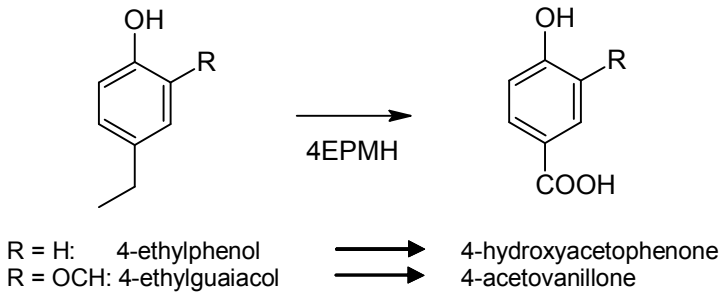


Figure 2.2. Breakdown of ethylphenols by 4EPMH.

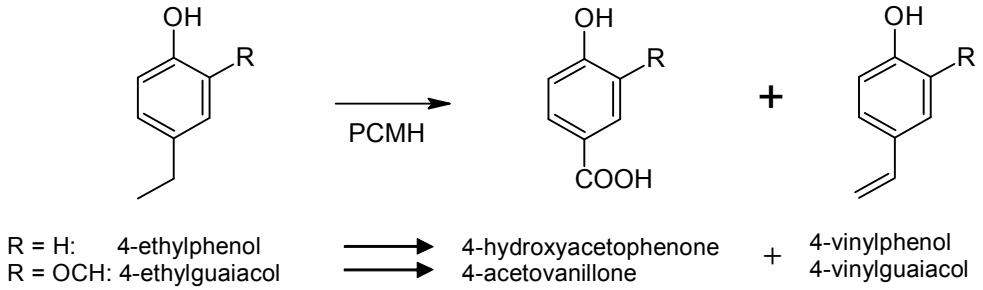


Figure 2.3. Breakdown of ethylphenols by PCMH.

5.3 Other compounds potentially associated with Brett character

Although volatile phenols are the compounds most commonly associated with Brett character, they are not the only compounds with sensory implications that have been linked to the metabolism of *Brettanomyces* in wines. Licker *et al*. (1999) reported a study into what they describe as the "larger picture of Brett character". Their study investigated three wines obtained from a winery that were described by the winemaker as "no Brett", "medium Brett" and "high Brett". The wines were shown to contain different levels of 4-ethylphenol in relation to their described level of Brett character. Sensory profiling of these wines showed that the higher "Brett" wines had more intense horse sweat, rubber, band-aid™ and plastic aromas, whereas the "no Brett" wine was dominated by aromas like spicy, earthy, woody, fruity and floral. This seems to indicate that the effect of *Brettanomyces* spoilage is not just the addition of some (seemingly negative) flavours such as band-aid, and horse-sweat, but also the suppression of other (often positive) wine flavours like fruity and floral.

GC-O coupled with Charm analysis (described by Acree *et al*., 1984) performed in the same study revealed that certain compounds other than the volatile phenols appear to add to the sensory effect that is known as Brett character. These included, in decreasing order of importance (according to the study) isovaleric acid, an unknown compound, 2-phenyl ethanol,

ethyl decanoate, cis-2-nonenal, guaiacol, 4-ethylphenol and trans-2-nonenal. The authors described their findings as a "snapshot" of the larger picture of Brett character.

The study done by Fugelsang and Zoecklein (2003) contradicts, some and confirms other findings of Chatonnet *et al.* (1992) and Licker *et al.* (1999). In this study a specific wine was inoculated with different strains of *Brettanomyces bruxellensis* and the levels of key metabolites were investigated. They found that although their wines had relatively low levels of 4-ethylphenol and 4-ethylguaiacol, they still had a distinct Brett character, which hints at synergistic sensory effect between different compounds. This study also indicated no significant difference in isovaleric acid levels between the control wines and wine inoculated with *Brettanomyces bruxellensis.* This suggests that isovaleric acid may not be the most important odorant associated with Brett character because not all strains of *Brettanomyces bruxellensis* produce this compound.

Romano *et al.* (2008, 2009) shed more light on the role of isovaleric acid in Brett character. Upon inoculating wines with a strain of *Brettanomyces bruxellensis*, it was found that the strain produced significant amounts of compounds other than volatile phenols, including carboxylic acids (acetic acid, isobutyric acid and isovaleric acid), short chain fatty acids (hexanoic acid and octanoic acid) and ethyl esters (ethyl octanoate). In a follow-up study (Romano *et al.*, 2009) no correlation was found between ethylphenol levels in commercial wines and their degree of sensory "Brettyness", as at least a third of the wines surveyed that did not have a noticeable degree of Brett character, but contained ethyl phenols at levels above the cumulative sensory threshold of 426 µg/L (Chatonnet *et al.*, 1992). A significant difference was also found between wines inoculated with *Brettanomyces bruxellensis* and the same wines spiked with corresponding amounts of 4-vinylphenol, 4-vinylguiacol, 4-ethylphenol and 4-ethylguaiacol, indicating that other volatile compounds have a detectable impact on wines spoiled by *Brettanomyces bruxellensis*. Further chemical investigation revealed a strong correlation between the production of isobutyric and isovaleric acid and that of ethylphenols, indicating that these compounds could possibly be considered markers for Brett character. Isovaleric and isobutyric acids are formed from L-leucine and L-valine respectively, as shown in Figure 2.4. The detection threshold of ethylphenols in combination with isovaleric and isobutyric acids were, however, found to be three times higher than that of the ethylphenols by themselves. The masking sensory effect of these acids may be due to the fact that their "rancid" and "sweaty" descriptors are quite similar to some of the descriptors usually used for wines with Brett character. This relationship, which could be either a linguistic confusion or a sensory interaction, is something which warrants investigation.

This already complex picture is complicated even further by other findings. A study done by Ferreira *et al*., (2006) on the kinetics of aroma extraction during aging in oak wood, showed that certain chemical compounds do not follow "logical" extraction patterns and suggests that these phenomena happen due to biochemical action on the oak wood. The levels of 4-

propylguaiacol extracted from oak wood were found to be positively correlated with higher levels 4-ethylphenol and 4-ethylguaiacol, which are the two compounds most commonly associated with Brett character. However, this correlation has yet to be confirmed.

The fact that *Brettanomyces* displays β-glucosidase activity may also impact on wine flavour. The enzymatic liberation of glycoside hydrolysis products may produce aroma, flavour and colour changes, which influences wine. The hydrolysis of glycosides may also increase varietal aroma and flavour in a wine, which would indicate that *Brettanomyces* has a positive effect on wine quality. However, the activity of these enzymes in fermentative environments is limited (Mansfield *et al.*, 2002). *Brettanomyces* also produces small amounts of acetaldehyde and ethyl acetate during aerobic fermentation (Ciani *et al.,* 2003). Acetaldehyde has sherry/nutty/bruised apple descriptors, and ethyl acetate contributes nail polish/fruity aromas to wine (Swiegers *et al.*, 2005). These compounds further add complexity to sensory character that may be produced by *Brettanomyces* spoilage of wine.

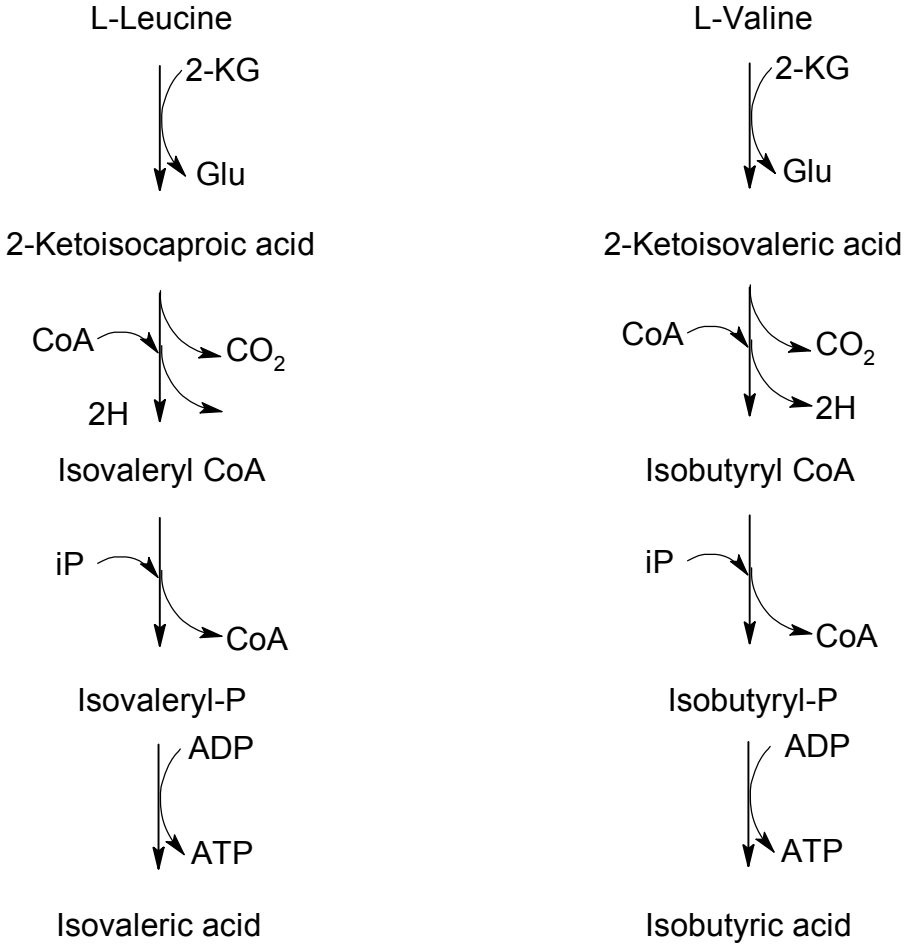


Figure 2.4. Production pathway of isovaleric and isobutyric acid from L-leucine and L-Valine respectively (Harwood & Canale-Parola, 1981).

6 FACTORS INFLUENCING LEVELS OF VOLATILE PHENOLS IN WINE

6.1 Factors influencing volatile phenol synthesis

It is estimated that a *Brettanomyces* cell count of approximately 10^5 cells/mL is required to trigger the production of ethylphenols in red wines (Fugelsang & Zoecklein, 2003). As previously mentioned, although higher levels of volatile phenols are produced later in the winemaking process, when higher numbers of cells are present (Renouf *et al*., 2007), cell numbers are not directly related to volatile phenol production (Fugelsang & Zoecklein, 2003). Ethylphenol production is also influenced by the type of strain present (Fugelsang & Zoecklein, 2003; Conterno *et al*., 2006). Ethylphenols are produced in wine during the lag phase of cell growth, and is inhibited by a lower pH (Romano *et al*., 2008).

The production of ethylphenols is also influenced by the availability of their hydrocinnamic acid precursors in the medium, and is proportional to the size of the *Brettanomyces*/*Dekkera* population in the wine (Saurez *et al*., 2007). Higher amounts of volatile phenols are produced at lower alcohol concentrations and higher temperatures (Gerbaux *et al*., 2002; Saurez *et al*., 2007). Heating must at the end of maceration also results in higher levels of volatile phenols, as does the use of extraction enzymes and some clarification enzymes during winemaking. Many commercial enzymes contain cinnamyl-esterase, which releases hydrocinnamic acids, leading to higher volatile phenol production (Gerbaux *et al*., 2002). *Aspergillus* moulds present on grape skins, and therefore in the must, also contain these esterases – which release phenolic acids bound to tartaric acids – and can therefore increase the free phenolic acid content, leading to higher levels of volatile phenols (Shinohara *et al*., 2000).

6.2 Volatile phenol sorption

Apart from breakdown to 4-hydroxyacetophenone, 4-acetovanillone, 4-vinylphenol and 4-vinylguaiacol, levels of 4-ethylphenol and 4-ethylguaiacol in wine can also be decreased by means of slow partitioning into oak wood and lees (Chassagne *et al.*, 2005; Barrerera-Garcia *et al*., 2006; Rayne & Eggers, 2007; Jiménez-Moreno & Ancín-Azpilicueta, 2009). This, along with the enzymatic breakdown of ethylphenols, results in what is referred to as a “Brett peak”, where maximum levels of ethylphenols can be found during the summer months, with a decrease during autumn. Samples taken and subjected to chemical or sensory analysis on either side of this “Brett peak” may result in a false negative result for *Brettanomyces* infection.

At typical wine pH values, the hydroxyl groups of 4-ethylphenol and 4-ethylguaiacol are not dissociated, which reduces the hydrophilic nature of the compounds, making them more

likely to associate with organic matter in solution (colloidal or dissolved tannins) or on surfaces, such as oak barrels (Rayne & Eggers, 2007). Barrera-Garcia *et al*. (2006) reported that the sorption of 4-ethylguaiacol and 4-ethylphenol into oak barrels occurs in two phases, with an initial fast sorption onto the wood surface during the first day, followed by a slower diffusion between the second and the eighth days. Sorption reached its peak at approximately 1000 mg of analyte per kg of wood, which is well above the typical amount of ethylphenols found in wines. The sorption of volatile phenols into yeast lees added to wine has been reported to reach a maximum at approximately 3 hours of contact. The sorption is accelerated by stirring of the wine (Chassagne *et al*., 2005; Jiménez-Moreno & Ancín-Azpilicueta, 2009).

The levels of 4-ethylphenol and 4-ethylguaiacol in a wine can also be reduced through a method combining reverse osmosis and adsorption (Ugarte *et al*., 2005). This method uses tangential-flow filtration equipment, which consists of a membrane filter, a hydrophobic adsorbent resin, and a reverse-osmosis feed tank. Although this method does not have a significant effect on colour, anthocyanins or tannins, a significant loss of esters like ethyl hexanoate and ethyl octanoate has been reported. These compounds are responsible for apple/banana and pineapple/pear descriptors. However, sensory analysis of wine treated with this method showed a higher level of fruitiness than an untreated wine. This substantiates the theory that the volatile phenols suppress fruity character in wines (Ugarte *et al*., 2005).

7 SENSORY AND CHEMICAL ANALYSIS METHODOLOGIES

7.1 Sensory methodologies associated with analysing Brett character

There are several factors that complicate sensory studies regarding Brett character in wines. The first is sample selection. The development of “natural” Brett character usually happens over a period of several months or years (Saurez *et al*., 2007), and simulating this in a laboratory environment is rarely practical. Spiking of wines with chemical compounds known or suspected to be responsible for Brett character also “confounds” some information, as chemicals may have sensory interactions that are not well known (Atanasova *et al*., 2005). Many studies select samples by means of informal sensory analysis, which usually involves tasting and selecting the wines that have the highest degree of Brett character. However, this approach has the subjectivity of the researcher selecting the samples built into it, as the selection depends on what they consider to be Brett character (Licker *et al*., 1999; Romano *et al*., 2009). Another issue is that methodologies for sensory analysis of wines differ greatly, with very few studies employing standardised sensory methodologies, which makes results difficult to compare. The fact that Brett character is associated with barrel aging, and that suppression of inherent fruitiness is one of its effects, also complicates the sensory analysis. Woody odorants, even

when below threshold levels, are known to suppress fruity odorants (Atanasova *et al*., 2005), and confusion by judges also occurs between odours of the same family (like woody and phenolic) (Escudero, 2007), making "true" descriptive analysis even more complicated.

When performing sensory analysis on a wine taint such as Brett character, there are several strategies that can be followed. These strategies are loosely divided into analytical tests and hedonic tests, with different standard methodologies existing for both. These tests are outlined in Figure 2.5. Only the tests in this figure relevant to dealing with taints will be discussed further.

7.1.1 Analytical tests

As can be seen in Figure 2.5, sensory analytical tests can be divided into difference tests and quantitative tests.

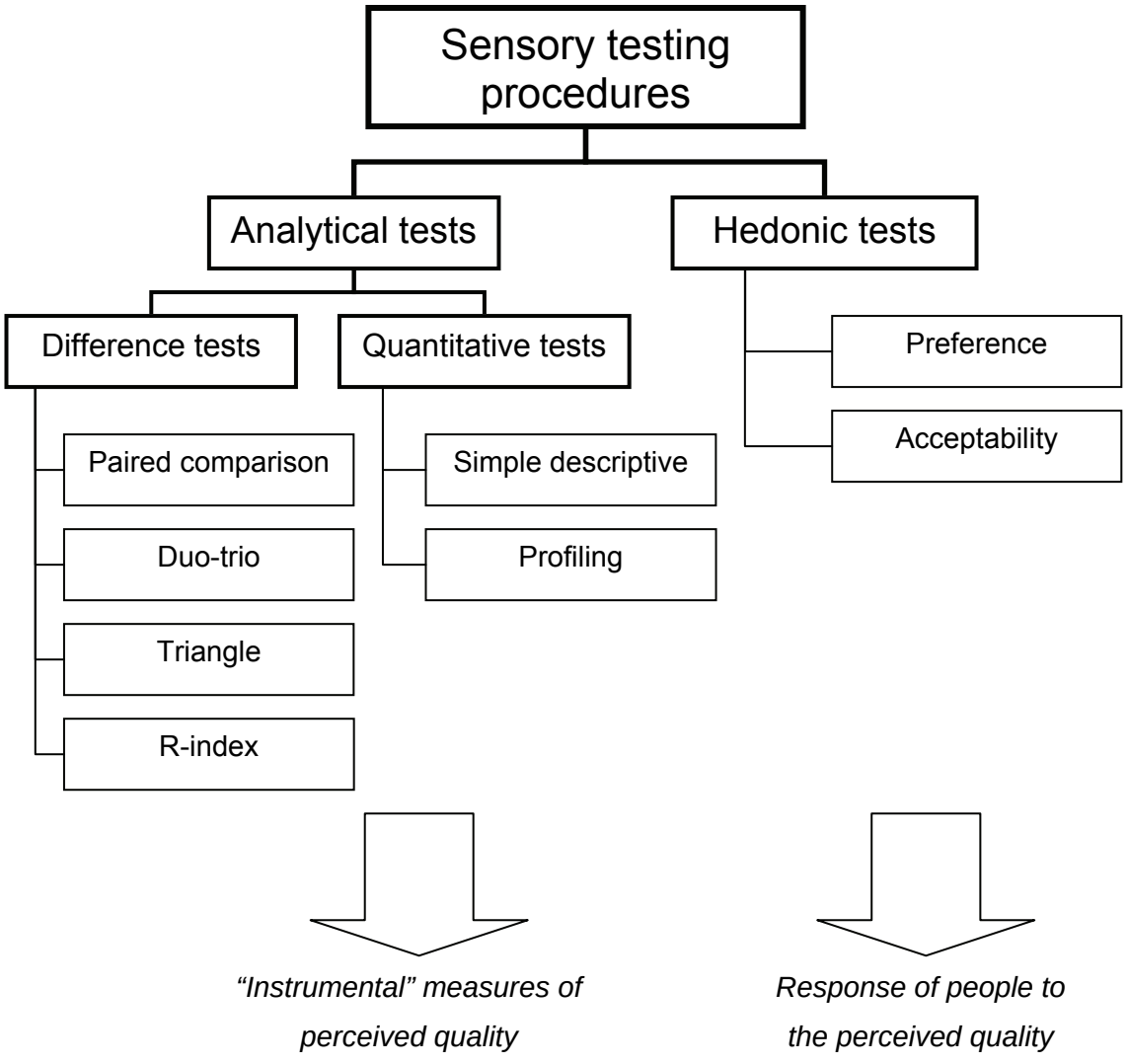


Figure 2.5. Outline of different types of sensory tests used for the analysis of taints (Kilcast, 2003).

Difference tests

The most common application of difference tests in the analysis of wine taints is for the determination of detection thresholds. Four types of difference tests exist, namely paired comparison, duo-trio tests, triangle tests and R-index tests.

The paired comparison test poses the question “Is there a difference between the two samples?” However, this test is not commonly used for the determination of detection thresholds, as it suffers from response bias (O’Mahony, 1995; Lawless & Heymann, 1998). Regardless, this test has been used by Hesford and Schneider (2004) for the determination of the detection threshold of 4-ethylcatechol and a value of 60 µg/L was obtained.

The duo-trio test consists of sets of three samples, of which one is set as a control. This test poses the question “Which of these samples is different from the control?” This method has been used to determine the detection threshold of 4-ethylcatechol (Larcher *et al*., 2008) and has been used by Fugelsang and Zoecklein (2003) to determine whether wines inoculated with *Brettanomyces* were different from a control.

Triangle tests also consist of sets of three samples, but in this test no control is specified, and the question posed is “Which of these samples is odd?” The three-alternative-forced choice (3-AFC) test is a special case of the triangle test where the difference between the samples is known. The 3-AFC has a higher probability of judges identifying the correct sample (O’Mahony, 1995). The International Organization for Standardisation (ISO) test for determining detection thresholds, as well as the one prescribed by the American Society for Testing and Materials (ASTM E-679) (ASTM, 1999) are both based on the 3-AFC test.

A traditional triangle test was used by Ferreira *et al*. (2000) for the determination of thresholds for several wine compounds. The ISO 13301 has been used for the determination of detection thresholds of volatile phenols in wine (Romano *et al*., 2009), as well as trichloroanisole (TCA) (Mazzoleni & Maggi, 2007). The ASTM test has been used for determining the detection threshold of oak lactones (Brown *et al*., 2006), diacetyl (Martineau *et al*., 1995) rotudone (Wood *et al*., 2008) and 3-isopropyl-2-methoxypyrazine (Pickering *et al*., 2007). The widespread use of the ASTM method can be ascribed to the fact that it is a standardised method which is commonly accepted as correct.

A final type of difference testing is the R-type test. The samples to be tested are compared to a standard and rated in one of four categories. When performing this type of difference testing, these categories are “standard”, “perhaps standard”, “perhaps not standard”, and “not standard”. The results are expressed in terms of R-indices, which represent probability values of correct discrimination (Lawless & Heymann, 1998; Kilcast, 2003). This test is not commonly used, but has been applied for the determination of detection thresholds of caffeine (Robinson *et al*., 2005) and the determination of off-flavour development time in beef (An *et al*., 2009). To date, this method has not been used for the analysis of wine.

Regardless of which type of difference testing is involved when detection thresholds are determined, the medium in which the detection threshold is determined is of utmost importance. Detection thresholds determined in wine are usually significantly different to those determined in model solution or water (Le Berre *et al.,* 2007). The wine type and style also influences the detection threshold (Martineau *et al*, 1995; Brown *et al.*, 2006). This makes it difficult to compare detection thresholds reported by different authors.

As previously mentioned, Chatonnet *et al.* (1992) determined the olfactory perception threshold of volatile phenols in water, model solution and red wines. These perception thresholds correspond to the minimum concentration at which 50% of a 70 person jury failed to taste the difference from a control. A similar test has recently been used for the determination of volatile phenols in olive oil (Vichi *et al*., 2009). Although this test seems simple and reliable enough, the detection threshold determined in such a manner is arbitrary and empirical, as there is no scientific basis for such a test (Lawless & Heymann, 1998). The perception thresholds determined by Chatonnet *et al*. (1992) for 4-ethylphenol and 4-ethylguaiacol in water were 130 and 25 µg/L, respectively, and in model solution 440 and 47 µg/L, respectively. The perception threshold of 4-ethylphenol in red wine was determined to be 605 µg/L, while that of 4-ethylguaiacol was 110 µg/L. The arbitrary method of determination is particularly important in this case as the detection thresholds determined by Chatonnet *et al*. (1992) are commonly cited as detection thresholds for these compounds.

Quantitative tests

Quantitative tests are used in order to define differences between different products, and can be used to define the differences between tainted and non-tainted wines. Quantification can occur either on a category scale or a line scale, which can be either unipolar or bipolar. Category scales use a defined number of boxes and the scale ends and intermediate points may be given verbal descriptions. Line scales consist of a line that usually only has verbal anchors at the ends. Reference points like samples or reference standards may be added to these scales. A unipolar scale has 0 at one end and the attribute at the other end. On the other hand, bipolar scales have opposite attributes at different ends (Lawless & Heymann, 1998; Kilcast, 2003). However, bipolar scales are not commonly used for quality assessment. Reference standards for profiling of wine have been developed (Noble *et al*., 1987) and these assist in quantitative tests in terms of defining differences and anchoring unstructured line scales. They are also used extensively for training of panelists.

Simple descriptive tests are used to quantify a simple well-defined characteristic. Quantitative descriptive analysis (QDA) is more complex than this, as several different attributes are profiled at the same time (Lawless & Heymann, 1998; Kilcast, 2003). This method has been used for the profiling of Brett wines (Wirz *et al*. 2004), wines at different temperatures spiked

with 4-ethylphenol (Cliff & King, 2009), as well as for other taints (Pickering *et al*., 2008). Modifications of this method have been used for profiling Brett character in several other instances (Etiévant *et al*., 1988; Licker *et al*, 1999; Ugarte *et al*., 2005).

Etiévant *et al.* (1989) performed descriptive analysis on a range of wines, with specific focus on how 4-ethylphenol and 4-ethylguaiacol affect the sensory profile of the wine. The data were, however, converted to a ranking and analysed nonparametrically using the Friedman test. This same method has been employed by other authors (Ugarte *et al*., 2005). Although this method compensates for variation between judges, a fair amount of sensory information can be lost.

In the study by Licker *et al*. (1999) mentioned in Section 5.3, three Cabernet Sauvignon wines that were identified by their winemaker as having a "no Brett", "medium Brett" and "high Brett" character were obtained from the same cellar. QDA was performed on these three wines in combination with Gas Chromatography-Olfactometry (GC-O) and Charm analysis. Charm analysis is a technique which attempts to quantify odour intensities in GC-O by means of repeating the GC-O analysis of a sample at successive dilutions. Judges are expected to respond when an odour appears, and when an odour stops appearing. A Charm response chromatogram is produced by plotting the retention index against *c*, a value related to the dilution factor and the number of judges that responded to the presence of an odour ($c = d^{n-1}$, where d is the dilution factor and n the number of coincident responses). An algorithm then provides a measure of sensory intensity (Acree *et al*., 1984).

In the latter study the QDA allowed the researchers to obtain a detailed profile of the different wines, allowing them to show that there is a distinct increase in plastic, horse sweat and band-aid™ aromas in the Brett wines, whilst the "no Brett" wine was predominated by fruity, floral, spicy, earthy and woody aromas. From these data the conclusion was drawn that the latter aromas were suppressed by Brett character. The combination of this data with the data obtained from GC-O allowed the researchers to identify a number of different compounds that contribute to Brett character that had previously not been considered.

7.1.2 Hedonic tests

The traditional method for the determination of the degree of liking of a wine is the nine-point hedonic scale. When this method is used, the consumer is asked to indicate which term best describes his/her attitude towards the products being tasted using the scale with the following nine categories (Lawless & Heymann, 1998): *9 = Like extremely; 8 = Like very much; 7 = Like moderately; 6 = Like slightly; 5 = Neither like nor dislike; 4 = Dislike slightly; 3 = Dislike moderately; 2 = Dislike very much* and *1 = Dislike extremely.* However, the performance of preference testing for tainted wines usually takes the form of consumer rejection thresholds, in which consumers have to identify which of a pair of samples (one containing the taint and one

not containing the taint) they prefer more. This method is generally recommended for use with tainted products (Kilcast, 2003) and has been employed for TCA (Prescott *et al*., 2005; Teixeira *et al*., 2006) and 1,8-cineole (eucalyptol) (Saliba *et al*., 2009). However, this approach tests preference between, and not rejection of, a tainted sample, and does therefore not necessarily provide relevant information. Such tests also ignore the inherent differences that exist between individual consumers and groups of consumers.

Two non-standard methodologies have been used for hedonic testing, although both attempt to set up consumer rejection thresholds. A test was done by Eteiévant *et al.* (1989) to determine the preferred level of 4-ethylphenol in wine. This was done by adjusting the 4-ethylphenol content of an existing wine to 8 concentrations ranging from 860 to 3880 µg/L , and supplying all judges with a reference sample containing an 12% ethanolic solution of 4-ethylphenol. A 100 mm line scale was used, with "No reference odour" indicated at the left of the line, and "Intensity of reference odour just right" indicated at 50 mm. Assessors were instructed to taste the wine and indicate their opinion about the reference flavour level by marking a point on the scale that best describes their opinion about the wine. In other words, if an assessor could not detect the reference odour, a mark would be made at 0 mm; if an assessor could detect the odour but felt that it was present at a too low concentration, a mark would be made between 0 mm and 50 mm. If the odour was at exactly the concentration that the assessor preferred, a mark would be made at 50 mm, and if this odour was found to be excessive, a mark would be made between 50 and 100 mm. A "mean ideal concentration" of 4-ethylphenol was determined for each assessor, and the overall mean was found to be 1800 µg/L, with the 95% confidence levels indicated to be between 1200 and 2400 µg/L. This test mixes the determination of detection thresholds with a preference test using a method most often used for descriptive analysis, which can make it unreliable.

A so-called "limit of preference threshold" was determined by Chatonnet *et al.* (1992) by asking 20 trained tasters which of two samples in a subset they preferred; one being spiked with the reference compound and one being "clean". The concentrations of the compounds that was rejected by 50% of the tasters was set as the limit of preference threshold. This threshold was determined as 620 µg/l for 4-ethylphenol and 140 µg/l for 4-ethylguaiacol. The limit of preference threshold for 4-ethylphenol and 4-ethylguaiacol combined in a ratio of 10:1 was found to be 426 µg/L. This value is widely used as a "rejection threshold" for wines containing volatile phenols. Although this test utilises an accepted methodology, it uses trained tasters, which are not recommended for preference test (Lawless & Heymann, 1998). The sample size of 20 judges is also too small to determine preference of a population (Kilcast, 2003). For this reason, the validity of the value and the common use of this value as rejection criterion should be questioned.

Although the determination of consumer rejection thresholds is the accepted and referred method of hedonic testing of tainted wines (Kilcast, 2003; Prescott *et al*., 2005; Teixeira

et al., 2006; Saliba *et al*., 2009), it may not be the most appropriate method for hedonic testing of wines tainted with Brett character. This is because Brett character is seen by some people to add complexity to wines, and therefore some "tainted" wines may be more acceptable to consumers than non-tainted wines.

7.1.3 Testing for association using statistical analyses

Quantitative sensory data can be combined with chemical data by means of multivariate statistics, which allows for more direct and accurate interpretation of the relationship between chemical and sensory data (Marias *et al*., 1979; Noble & Ebeler, 2002; Chung *et al*., 2003; Boselli *et al*., 2004; Genovese *et al*., 2007). The simplest multivariate method is Principal Component Analysis (PCA), which reduces multidimensional datasets to orthogonal "principal components". These components are modelled in such a way that the first component is in the direction describing most of the variation in the dataset, and each successive orthogonal component explains successively less of the variation. An overview of the most important aspects of complex datasets can therefore be given by plotting principal components against each other. On a PCA plot, samples are plotted as "scores", and variables are plotted as "loadings". On such a plot, loadings that associate positively are in the same direction, and loadings that associate negatively are in opposite directions. Loadings that are perpendicular have no association with one another. Similarly, scores that are similar associate and scores that are dissimilar associate negatively (Esbensen, 2002). The underlying principles of PCA are common to many multivariate methods.

When it comes to the combination of sensory and chemical data, two general sets of multivariate methods are used; namely symmetric and asymmetric methods. Symmetric methods include General Proscrutes Analysis (GPA) and Canonical Correlation Analysis (CCA) (Dijksterhuis, 1995). These methods treat both datasets the same, and are more interested in finding relationships between the different datasets than predicting the scores and loadings of one dataset from the scores and loading of the other dataset, as is the case with assymetric methods. For example in GPA, the "one (PCA) space is rotated, reflected, and stretched or shrunk (scaled) to optimally match the second space" (Noble & Ebeler, 2002). GPA is considered a suitable method for understanding the relationships between chemical composition and sensory attributes (Chung *et al*., 2003).

Asymmetric methods attempt to predict the scores and loading of one dataset from the scores and loadings of another dataset. Examples of commonly used asymmetric methods are partial least squares regression methods (PLS, PLS2) and principal component regression (PCR) (Dijksterhuis, 1995). PLS is a "soft modelling" technique that extracts "factors" or latent variables. These factors are linear combinations of one set of variables that predict a large amount of variation in another set of variables (Noble & Ebeler, 2002). PCR, on the other hand,

is a multiple linear regression performed on principal component scores (Esbensen, 2002). Although a discussion of the suitability of these methods are beyond the scope of this work, it can be noted that PLS appears to be more commonly used than PCR for the modelling of sensory data (Noble & Ebeler, 2002; Frøst & Noble, 2002; Chung, 2003).

Asymmetric multivariate methods form the basis of electronic nose and electronic tongue technologies (Buratti *et al*., 2007), which have also been investigated for identification of Brett character in wine (Cyncar *et al*., 2007). This method is discussed in Section 7.2.

Preference mapping is a method that can be used to correlate the preference of different groups of consumers to the sensory and/or chemical qualities of food products and beverages (Tuorila & Monteleone, 2009). Two basic types of preference mapping exist, namely internal preference mapping and external preference mapping (Anon, 2009). In their review on preference mapping techniques, Meilgaard *et al.* (2007), however, designates PLS mapping to be a third type of preference map. Internal preference mapping (the MDPREF procedure) is used to summarise the liking of a large group of consumers to various products, without taking any of the intrinsic properties of the products into account. External preference mapping (the PREFMAP procedure) involves relating the preference of consumers to specific characteristics of products like sensory properties, physio-chemical properties or economic attributes (Anon, 2009). PCA is the underlying multivariate technique for both these methods. PLS mapping is a direct application of partial least squares regression (PLS-2), with the sensory or chemical data in the X space, and the consumer data in the Y (predictive) space (Meilgaard *et al*., 2007).

Preference mapping has been used for wine (Frøst & Noble, 2002), several food products (apples (Thybo *et al*., 2003), yogurt, pudding (Elmore *et al*., 1999) and beverages (tea (Cho *et al*., 2005) coffee (Geel *et al*., 2005), and rice wine (Lee & Lee, 2008)). This method has not yet been used for the analysis of wine taints, but has immense potential for application to a problem such as Brett character.

7.2 Chemical methodologies for analysing compounds associated with Brett character

It is essential to accurately determine the concentrations of the relevant compounds associated with *Brettanomyces* in any study attempting to study this phenomenon in detail.

The most common method for the determination of the ethylphenols is gas chromatography, which can be coupled to either a flame ionization detector (FID) (Martorell *et al*., 2002; Monje *et al*., 2003), mass spectrometry (MS) (Etiévant, 1981) or tandem mass spectrometry (MS-MS) (Pizarro *et al*., 2007). Mass spectrometry appears to be more popular than FID. Tandem mass spectroscopy provides even better sensitivity, but the instrumentation is more expensive and not as commonly available.

In terms of sample preparation, liquid-liquid extraction (LLE) is frequently used. Solvents include dichloromethane (Chatonnet *et al*., 1992; 1993), diethyl ether-pentane (Pollnitz *et al*., 2000) or ether-hexane (Rodriguez *et al*. 2001; Dias *et al*. 2003; Martorell *et al*., 2006). Dispersive liquid-liquid microextraction (DLLME) has also been investigated as an alternative sample preparation method (Fariña *et al*, 2007). This method uses two solvents – an extractor solvent which has a higher density than the sample and has an affinity for the compounds in question, and a disperser solvent which is miscible with both the sample and the extractor solvent. The disperser solvent acts as a "carrier" between the sample and the extractor solvent. An example of such a solvent system is carbon tetrachloride (extractor solvent) and acetone (disperser solvent). Although this method produced relatively high limits of quantification and detection (see Table 2.3), its small sample volume, short extraction time (6 minutes) and low solvent usage, all which make it less expensive than LLE, are major advantages.

Stir bar sorptive extraction has also been investigated (Díez *et al*., 2004), but the limits of detection and quantification reported were significantly higher than many other methods, and, in the case of 4-ethylguaiacol (159 µg/L) much higher than the sensory detection threshold (33 µg/L). This method also has a rather extensive extraction time (60 minutes).

Significant research has been done into the use of sorptive extraction methods for the analysis of ethylphenols. These include headspace solid-phase microextraction (HS-SPME) (Monje *et al*., 2002; Martorell *et al*., 2002; Carrilo *et al*., 2006; Botou & Chatonnet, 2007) and multiple headspace microextraction (Pizarro *et al*., 2007). A general advantage of these methods is that they require little sample preparation and do not require a solvent. A common choice of fibre is divinylbenzene-caboxen-poly(dimethylsiloxane) (DVD/CAR/PDMS) (Carillo *et al*., 2006; Carillo & Tena, 2007; Botou & Chatonnet, 2007), although polydimethylsiloxane (PDMS) (Martorell *et al*., 2002) and polyacrylate (Monje *et al*., 2002) have also been used with success. Pizarro *et al*. (2007) reported severe matrix effects for MS-SPME, which may have been due to the use of a carbowax/divinylbenzene fibre. HS-SPME coupled to GC-MS also generally gives low LOD's and LOQ's (see Table 2.3). In most cases, however, matrix effects are significant, which means that standard addition must be used for quantification, which is extremely time-consuming. (MHS-SPME does not have this disadvantage (Pizarro *et al*., 2007), but requires several extraction steps, which is also time-consuming.)

Derivitisation prior to extraction has also been applied. Such a derivitisation step is necessary for the determination of 4-ethylcatechol, as it highly polar and non-volatile, and causes peak tailing even when a polar GC column is used. This is overcome by for example acetylation of the compound through the addition of acetic anhydride (Carillo & Tena, 2007). Although this method is successful for the detection of 4-ethylcatechol, its limits of quantification an detection are approximately double those found using similar methods (Carillo *et al*., 2006).

Table 2.3. Recent methods used for determination of ethylphenols in wines showing limits of detection (LOD) and limits of quantification (LOQ). All values are in µg/L.

Method	LOD 4-EP	LOD 4-EG	LOD 4-EC	LOQ 4-EP	LOQ 4-EG	LOQ 4-EC	Authors
DLLME[a] GC-MS	44	28	-	147	95	-	Fariña *et al.,* 2007
SBSE[b] GC-MS	6	159	-	21	529	-	Díez *et al*., 2004
HS-SPME[c] GC-FID	2	1	-	5	5	-	Martorell *et al*., 2002
HS-SPME GC-MS	7	1	-	15	2	-	Carillo *et al*., 2006
HS-SPME GC-MS	11.5	3.8	-	25.1	9.1	-	Botou & Chatonnet, 2007
HS-SPME GC-MS	17	2	4	30	3	6	Carillo & Tena, 2007
MHS-SPME[d] GC-MS/MS	0.06	0.06	-	0.20	0.18	-	Pizarro *et al*., 2007
LC-MS/MS	10	10	-	50	50	-	Caboni *et al*., 2007
HPLC-DAD	10	10	-	50	50	-	Caboni *et al*., 2007
HPLC- fluorescence	1	10	-	5	50	-	Caboni *et al*., 2007
HPLC-CEAD[e]	1.30	1.57	-	2.59	3.13	-	Larcher *et al*., 2007
HPLC-CEAD	1.30	1.57	0.33	2.59	3.13	1.1	Larcher *et al*., 2008

[a] Dispersive liquid-liquid microextraction [b] Stir bar sorptive extraction [c] Head-space solid-phase microextraction [d] Multiple head-space solid-phase microextraction [e] Coulometric array detector

There has also been recent interest in liquid chromatographic methods for the determination of the ethylphenols. These methods require no sample preparation and have the significant advantage that they are more suitable for the determination of 4-ethylcatechol than GC-MS. A HPLC-Coulometric method for the determination of 4-ethylphenol and 4-ethylguaiacol was developed by Larcher *et al*. (2007), which was subsequently adapted for the analysis of 4-ethylcatechol (Larcher *et al*., 2008). As can be seen in Table 2.3, these methods have relatively low limits of detection and quantification, especially when compared to GC-MS preceded with derivitisation (Carillo & Tena, 2007).

Caboni *et al*. (2007) developed a LC-MS/MS method as well as a HPLC-diode array method and a HPLC-fluorescence method. However, the limits of quantification for these methods are high when compared to GC-MS, and especially high when compared to GC-MS-MS (Pizarro *et al*., 2007). The main advantage of this method is therefore that no sample preparation is required. An HPLC-DAD method has also recently been developed for the analysis of 4-vinylcatechol, the precursor of 4-ethylcatechol (Hisomoto *et al*., 2009). The other ethylphenols could also be detected with this method.

Cyncar *et al.* (2007) tested the feasibility of using an MS electronic nose method to discriminate between commercial wines according to their 4-ethylphenol levels. The categories of high (higher than 500 µg/L), medium (between 500 and 200 µg/L), and low (lower than 100 µg/L) were tested and the method was found to have a classification rate of 67%. This method might be able to produce at least a 40% reduction in cost as compared to headspace GC-MS or sensory analysis methods. The method also has potential as a routine method for wine quality monitoring. Further studies on this instrument (Berna *et al*., 2008) found that concentrations of

4-ethylphenol of higher than 20 µg/L could be reliably estimated. In spite of this improvement, this technique is not developed to a level where it is applicable for routine wine monitoring.

8 SUMMARY

Brettanomyces forms part of the incredibly complex microbiology of wine. Although this micro-organism has been known for several decades, there are still several unanswered questions regarding its sensory effects in red wine, particularly South African red wine. The first of these is what the potential effect of elevated levels of 4-ethylcatechol has in South African wines, and what the effects of other metabolites of *Brettanomyces* are on the sensory character of wine. Although attempts have been made to study these effects, more accurate answers could be obtained by using appropriate sensory research methodology, as well as combining sensory data with chemical data through multivariate statistics. A final area of question is how the South African wine consumer responds to wines spoiled by *Brettanomyces*, and whether or not these consumers find wine spoiled by this micro-organism objectionable.

The answers to these questions all relate to the chemical diagnosis of the sensory effect which is Brett character. The question remains at which levels the Brett-related spoilage compounds can be detected in wine, as well as at which levels these compounds become objectionable. Furthermore, the sensory effect of 4-ethylcatechol and its sensory interaction with the other Brett-related spoilage compounds has not yet been investigated. These two aspects are of utmost importance for the South African wine industry for several reasons. 4-ethylcatechol is not currently considered as an important Brett compound, and diagnostic analyses do not include this compound. However, Pinotage, a uniquely South African cultivar, contains exceedingly high quantities of caftaric acid and caffeic acid, making this cultivar more susceptible to high levels of this compound.

9 REFERENCES

Acree, T. E., Barnard, J. & Cunningham, D. G. (1984) A procedure for the sensory analysis of gas chromatographic effluents. *Food Chemistry*. **14**, 273 - 286.

An, S.R., Shim, S.D., Lee, J.Y., Hong, K. W & Lee, S. J. (2009) Investigation on beef quality Indicator of off-flavor development during storage (abstract only). *Korean Journal for Food Science of Animal Resources*. **29**, 325 - 333.

Anomynous (2009). What is Preference Mapping? [WWW document]. http://www.xlstat.com/en/support/tutorials/prefmap.htm. 29 October 2009.

Arriagada-Carrazana, J. P., Sáez-Navarette, C. & Bordeu, E. (2005) Membrane filtration effects on aromatic and phenolic quality of Cabernet Sauvignon wines. *Journal of Food Engineering*. **68**, 363 - 368.

ASTM (1997). Standard practice E 679-91. Determination of odor and taste thresholds by an ascending concentration series method of limits. Philadelphia, USA: American Society for Testing and Materials

Atanasova, B., Thomas-Danguim, T., Langious, D., Nickluas, S., Chabanet, C., & Etiévant, P. (2005) Perception of wine fruity and woody notes: influence of peri-threshold odorants. *Food Quality and Preference*. **16**, 504 - 510.

Barata, A., Caldiera, R., Botelheiro, R., Pagliara, D., Malfeito-Ferreira, M. & Loureiro, V. (2008) Survival patterns of *Dekkera bruxellensis* in wines and inhibitory effects of sulphur dioxide. *International Journal of Food Microbiology*. **121**, 201 - 207.

Barrera-García, V. D., Gougeon, R. D., Voilley, A. & Chassagne, D. (2006) Sorption behavior of volatile phenols at the oak wood/wine interface in a model system. *Journal of Agricultural and Food Chemistry*. **54**, 3982 - 3989.

Berna, A. Z., Trowell, S., Cynkar, W. & Cozzolino, D. (2008) Comparison of metal oxide-based electronic nose and mass spectrometry-based electronic nose for the prediction of red wine spoilage. *Journal of Agricultural and Food Chemistry*. **56**, 3238 - 3244.

Boselli, E., Boulton, R. B., Thorngate, J. H. & Frega, N. G. (2004) Chemical and sensory characterisation of DOC red wines from Marche (Italy) related to vintage and grape cultivars. *Journal of Agricultural and Food Chemistry*. **52**, 3843 - 3854.

Boutou, S. & Chatonnet, P. (2007) Rapid headspace solid-phase microextraction/gas chromatographic/mass spectrometric assay for the quantitative determination of some of the main odorants causing off-flavours in wine. *Journal of Chromatography A*. **1141**, 1 - 9 .

Brown, R. C., Sefton, M. A., Taylor, M. A., Taylor, D. K. & Elsey, G. M. (2006) An odour detection threshold determination of all four possible stereoisomers of oak lactone in a white and red wine. *Australian Journal of Grape and Wine Research*. **12**, 115 - 118.

Buratti, S., Ballabio, D., Benedetti, S. & Cosia, M. S. (2007) Prediction of Italian red wine sensorial descriptors from electronic nose, electronic tongue and spectrophotometric measurements by means of Genetic Algorithm regression models. *Food Chemistry.* **100**, 211 - 218.

Caboni, P., Sarais, G., Cabras, M. & Agoini, A. (2007) Determination of 4-ethylphenol and 4-ethylguiacol in wine by LC-MS-MS and HPLC-DAD-fluorescence. *Journal of Agricultural and Food Chemistry*. **55**, 7288 - 7293.

Campolongo, S., Rantsiou, K., Giordano, M., Gerbi, V. & Cocolin, L. (2009) *Brettanomyces bruxellensis:* incidence and diversity in Italian Wines as determined by molecular Methods. *American Journal of Enology and Viticulture*. **60**, 398.

Carillo, J. D., Garrido-López, A. & Tena, M. T (2006) Determination of volatile oak compounds in wine by headspace solid-phase microextraction and gas chromatography-mass spectrometry. *Journal of Chromatography A*. **1102**, 25 - 36.

Carrillo, J. D. & Tena, M. T. (2007) Determination of ethylphenols in wine by in situ derivitisation and headspace solid-phase microextraction-gas chromatography-mass spectrometry. *Analytical and Bioanalytical Chemistry*. **387**, 2547 - 2558.

Chassagne, D.M., Cuilloux-Benatier, M., Alexandre, H. & Voilley, A. (2005) Sorption of wine volatile phenols by yeast lees. *Food Chemistry*. **91**, 39 - 44.

Chatonnet, P., Dubourdieu, D. & Boidron, J. (1995) The influence of *Brettanomyces*/*Dekkera* sp. yeasts and lactic acid bacteria on the ethylphenol content of red wines. *American Journal of Enology and Viticulture*. **46**, 463 - 468.

Chatonnet, P., Dubourdieu, D., Boidron, J. & Lavigne, V. (1993) Synthesis of volatile phenols by *Saccharomyces cerivisae* in Wines. *Journal of the Science of Food and Agriculture*. **62**, 191 - 202.

Chatonnet, P., Dubourdieu, D., Boidron, J. & Pons, M. (1992) The origin of ethylphenols in wines. *Journal of the Science of Food and Agriculture*. **60**, 165 - 175.

Cho, H., Chung, S., Kim, H. & Kim, K. (2005) Effect of sensory characteristics and non-sensory factors on consumer liking of various canned tea products. *Journal of Food Science*. **70**, 532 - 538.

Chung, S., Heymann, H. & Grün, I. U. (2003) Application of GPA and PLSR in correlating sensory and chemical data sets. *Food Quality and Preference*. **14**, 485 - 495.

Ciani, M. & Ferraro, L. (1997) Role of oxygen on acetic acid producing by *Brettanomyces*/*Dekkera* in winemaking. *Journal of the Science of Food and Agriculture*. **75**, 489 - 495.

Ciani, M., Maccarelli, F. & Fatichenti, F. (2003) Growth and fermentation behavior of *Brettanomyces*/*Dekkera* yeast under different conditions of aerobiosis. *World Journal of Microbiology and Biotechnology*. **19,** 419 - 422.

Cliff, M. A. & King, M. C. (2009) Influence of serving temperature and wine type on perception of ethyl acetate and 4-ethyl phenol in wine. *Journal of Wine Research*. **20**, 45 - 52.

Conterno, L., Hesford, F. & Henick-Kling, T. (2005) Volatile phenols off-odor production by different strains of *Brettanomyces bruxellensis. American Journal of Enology and Viticulture*. **56**, 292A.

Cyncar, W., Cozzolino, D., Dambergs, B., Janik, L. & Gishen, M. (2007) Feasibilty study on the use of a head space mass spectrometry electronic nose (MS e_nose) to monitor red wine spoilage induced by *Brettanomyces* yeast. *Sensors and Actuators*. **124**, 167 - 171.

De Villiers, A., Majek, P., Lynen, F., Crouch, A., Lauer, H. & Sandra, P. (2005) Classification of South African red and white wines according to grape variety based on the non-coloured phenolic content. *European Food Research and Technology*. **221**, 520 - 528.

Dias, L., Pereira-da-Silva, S., Tavares, M., Malfeito-Ferreira, M. & Loureiro, V. (2003) Factors affecting the production of 4-ethylphenol by the yeast *Dekkera bruxellensis* in enological conditions. *Food Microbiology.* **20**, 377 - 384.

Díez, J., Domínguez, C., Guillén, D. A., Veas, R. & Barosso, C. G. (2004) Optimisation of stir bar sorptive extraction for the analysis of volatile phenols in wines. *Journal of Chromatography A*. **1025**, 263 - 267.

Dijksterhuis, G. (1995) Multivariate data analysis in sensory and consumer science: An overview of developments. *Trends in Food Science & Technology*. **6**, 206 - 211.

du Toit, W. J., Pretorius, I. S. & Lonvaud-Funel, A. (2005) The effect of sulphur dioxide and oxygen on the viability and culturabilty of a strain of *Acetobacter pasteurianus* and a strain of *Brettanomyces bruxellensis* isolated from wine. *Journal of Applied Microbiology*. **98**, 862 - 871.

Elmore, J. R., Heymann, H., Johnson, J. & Hewett, J. E. (1999) Preference mapping: relating acceptance of "creaminess" to a descriptive sensory map of a semi-solid. *Food Quality and Preference*. **10**, 465 - 475.

Esbensen, K. H. (2002) Multivariate Data Analysis, 5th ed. Osla, Norway: CAMO Process AS

Escudero, A., Campo, E., Fariña, L., Cacho, J. & Ferreira, V. (2007) Analytical characterization of the aroma of five premium red wines. Insights into the role of odor families as the concept of fruitiness of wines. *Journal of Agricultural and Food Chemistry*. **55**, 4501 - 4510.

Eteiévant, P. X. (1981) Volatile phenol determination in wine. *Journal of Agricultural and Food Chemistry*. **29**, 65 - 67.

Eteiévant, P. X., Issanchou, S. N., Marie, S., Ducruet, V. & Flanzy, C. (1989) Sensory impact of volatile phenols on red wine aroma: influence of carbonic maceration time and storage. *Sciences des aliments*. **9**, 19 - 31.

Farina, L., Boido, E., Carrau, F. & Dellacassa, E. (2007) Determination of volatile phenols in red wines by dispersive liquid-liquid microextraction and gas chromatography-mass spectrometry detection. *Journal of Chromatography A*, **1157**. 1157, 46 - 50.

Ferreira, V., Jaruta, I. & Cacho, J. (2006) Physiochemical model to interpret the kinetics of aroma extraction during wine aging in wood. Model limitations suggest the necessary existance of biochemical processes. *Journal of Agricultural and Food Chemistry*. **54**, 3047 - 3054.

Ferreira, V., López, R. & Cacho, J. F. (2000) Quantitative determination of the odorants of young red wines from different grape varieties*. Journal of the Science of Food and Agriculture*. **80**, 1659 - 1667.

Frøst, M. B. & Noble, A. C. (2002) Preliminary study on the effect of knowledge and sensory expertise on liking for red wines. *American Journal of Enology and Viticulture*. **53**, 275 - 284.

Fugelsang, K. C. & Zoeklein, B. W. (2003) Population dynamics and effects of *Brettanomyces bruxellensis* strains on Pinot noir (Vitis vinifera L.) wines. *American Journal of Enology and Viticulture*. **54**, 294 - 300.

Geel., L., Kinnear, M. & de Kock, H.L. (2005) Relating consumer preferences to sensory attributes of instant coffee. *Food Quality and Preference*. **16**, 237 - 244.

Gerbaux, V., Vincent, B. & Bertrand, A. (2002) Influence of maceration temperature and enzymes on the content of volatile phenols in Pinot noir wines. *American Journal of Enology and Viticulture*. **53**, 131.

Harwood C. S. & Canale-Parola, E. (1981) Adenosine-5'-triphosphate-yielding pathways of branched-chain amino acid fermentation by a marine spirochete. *Journal of Bacteriology*. **148**, 117 - 123.

Hesford, F. & Schneider, K. (2004) Discovery of a third ethylphenol contributing to *Brettanomyces* taint. *Obst- und Weinbau*. **140**, 11-13.

Hesford, F., Schneider, K., Porret, N. & Gafner, J. (2004) Identification and analysis of 4-ethyl catechol in wines tainted by *Brettanomyces* off-flavor. *American Journal of Enology and Viticulture*. **55**, 304A.

Hisomoto, M., Okuda, T., Nishimoto, S., Tani, K., Tachibana, M., Koizumi, H., Kiba, N. & Yokotsuka, K. (2009) Determination of 4-vinylcatechol in wine by HPLC-DAD coupled with flourescence detection. *American Journal of Enology and Viticulture*. **60**, 402.

Jiménez-Moreno, N. & Ancín-Azpilicueta, C (2009) Sorption of volatile phenols by yeast cell walls. *Internationl Journal of Wine Research*. **1**, 11-18.

Kelly, J. (2003) What is *Brettanomyces* and why are so many winemakers concerned about it? *The Australian & New Zealand Grapegrower & Winemaker*. **474**, 54 - 57.

Kilcast, D. (2003) *Sensory analytical methods in detecting taints and off-flavours in food*. IN: Taints and off-flavours in foods. (edited by B. Baigrie). Pp 5 - 30. Cambridge, England: Woodhead Publishing Ltd.

Larcher, R., Nicolini, G., Bertoldi, D. & Nardin, T. (2008) Determination of 4-ethylcatechol in wine by high-performance liquid chromatography-coulometric electrochemical array detection. Analytica Chimica Acta. **609**, 235 - 240.

Larcher, R., Nicolini, G., Puecher, C., Bertoldi, D., Moser, S. & Favaro, G. (2007) Determination of volatile phenols in wine using high-performace liquid chromatography with a coulometric array detector. *Analytica Chimica Acta*. **582**, 55 -60.

Lawless, H. T. & Heymann, H. (1998*). Sensory evaluation of food, principles and practice*. New York, USA: Chapman and Hall.

Le Berre, E., Atanasova, B., Langloi, Etiéviant, P. & Thamas-Danguin, T. (2007) Impact of ethanol on the perception of wine odorant mixtures. *Food Quality and Preference*. **18**, 901 - 908.

Lee, S. J. & Lee, K. G. (2008) Understanding consumer preferences for rice wines using sensory data. *Journal of the Science of Food and Agriculture*. **88**, 690 - 698.

Licker, J. L, Acree, T. E. & Henick-Kling, T. (1999) What is "Brett" (*Brettanomyces*) flavour? A preliminary investigation. In: *Chemistry of Wine Flavour. ACS Symposium Series* (edited by A. L. Waterhouse & S. E. Ebeler), Pp 96 - 115. Washington DC: American Chemical Society

Loureiro, V. & Malfeito-Ferreira, M. (2003) Spoilage yeasts in the wine industry. *International Journal of Food Microbiology*. **86**, 25 - 20.

Mansfield, A. K., Zoecklein, B. W. & Whinton, R. S. (2002) Quantification of glycosidase activity in selected strains of *Brettanomyces bruxellensis* and *Oenococcus oeni*. *American Journal of Enology and Viticulture*. **53**, 303 - 307.

Marais, J., Van Rooyen, P. C. & Du Plessis, C. S. (1979) Objective quality rating of Pinotage wine. *Vitis*. **18**, 31 - 39.

Martineau, B., Acree, T. E. & Henick-Kling, T. (1995) Effect of wine type on detection threshold for diacetyl. *Food Research International*. **28**, 139 - 143.

Martorell, N., Martí, M. P., Mestres, M., Busto, O. & Guasch, J. (2002) Determination of 4-ethylguaiacol and 4-ethylphenol in red wine using headspace-solid-phase microextraction-gas chromatograpgy. *Journal of Chromatography A*. **978**, 349 - 354.

Martorell, P., Barata, A., Malfeito-Ferreira, M., Fernández-Espinar, M. T., Loureiro, V. & Querol, A. (2006) Molecular typing of the yeast species *Dekkera bruxellensis* and *Pichia guilliermondii* recovered from wine related sources*. International Journal of Food Microbiology*. **106**, 79 - 84.

Mazzoleni, V. & Maggi, L. (2007) Effect of wine style on the perception of 2,4,6-trichloroanisole, a compound related to cork taint in wine. *Food Research International*. **40**, 649 - 699.

Millet, V. & Lonvaud-Funel, A. (2000) The viable but non-culturable state of wine micro-organisms during storage. *Letters in Applied Microbiology*. **30**, 136 - 141.

Monje, M., Privat, C., Gastine, V & Nepveu, F. (2002) Determination of ethylphenol compounds by headspace solid-phase microextraction in conjunction with gas chromatography and flame ionization detection. *Analytica Chimica Acta*. **458**, 111 -117.

Noble, A. C. & Ebeler, S. E. (2002) Use of Multivariate statistics in understanding wine flavour. *Food Reviews International*. **18**, 1 - 21.

Noble, A.C., Arnold, R.A., Buechsenstein, J., Leach, E. J., Schmidt, J. O. & Stern, P. M. (1987) Modification of a standardized system of wine aroma terminology. *American Journal of Enology and Viticulture*. **38**, 143 - 146.

Norris, L. (2004) Unraveling the mystery of *Brettanomyces* flavor. *American Journal of Enology and Viticulture*. **55**, 304A.

Oelofse, A., Pretorius, I. S., & du Toit, M. (2008) Significance of *Brettanomyces* and *Dekkera* during winemaking: A synoptic review. *South African Journal of Enology and Viticulture*. **29**, 128 - 144.

O'Mahony, M. (1995) Who told you the triangle test was simple?. *Food Quality and Preference*. **6**, 227 - 738.

Parish, M., Kelly, M. & Baldwin, G. (2003) Managing Brett. *Australian and new Zealand Wine industry Journal*. **18**, 12 - 15.

Pickering, G. J., Karthik, A., Inglis, D., Sears, M. & Ker, K. (2007) Determination of ortho- and retronasal detection thresholds for 2-isopropyl-3methoxypyrazine in wine. *Journal of Food Science*. **27**, 468 - 472.

Pickering, G. J., Spink, M., Kotserdis, Y., Inglis, D., Brindle, I. D., Sears, M. & Beh, A. L (2008) Yeast strain affects 3-isopropyl-2-methoxypyrazine concentration and sensory profile in Cabernet Sauvignon wine. *Australian Journal of Grape and Wine Research*. **14**, 230 - 237.

Pizarro, C., Pérez-del-Notario, N. & González-Sáiz, J. M. (2007) Determination of Brett character responsible compounds in wines by using multiple headspace solid-phase microextraction. *Journal of Chromatography A*. **1143**, 176 - 181.

Pollnitz, A. P., Pardon, K. H. & Sefton, M. A. (2000) Quantitative analysis of 4-ethylphenol and 4-ethylguaiacol in red wine. *Journal of Chromatography A*. **874**, 101 - 109.

Prescott, J., Norris, L., Kunst, M. & Kim, S. (2005) Estimating a "consumer rejection threshold" for cork taint in white wine. *Food Quality and Preference*. **16**, 345 - 349.

Rayne, S., & Eggers, N. (2007) Volatile phenols from *Brettanomyces* in barreled red wines: are they stable? And where do they reside? *The Australian & New Zealand Grapegrower & Winemaker*. **522**, 64 - 69.

Renouf, V., Louvaud-Funel, A. & Coulon, J. (2007) The origin of *Brettanomyces bruxellensis* in wines: A review. *Journal Internationale de Science de Vigne et Vin*. **41**, 161 - 173.

Renouf, V., Perello, M., de Revel, G. & Lonvaud-Funel, A. (2007) Survival of wine microorganisms in the bottle during storage. *American Journal of Enology and Viticulture*. **58**, 379 - 386.

Robinson, K. M., Klien, B. P. & Lee, S. -Y. (2005) Utilizing the R-index measure for threshold testing in model caffeine solutions. *Food Quality and Preference*. **16**, 283 - 289.

Rodrigues, N., Gonçalves, G., Pereira-da-Silva, S., Malfeito-Ferreira, M. & Loureiro, V. (2001) Development and use of a new medium to detect yeasts of the genera *Dekkera/Brettanomyces*. *Journal of Applied Microbiology*. **90**, 588 - 599.

Romano, A., Perello, M. C., de Revel, G. & Lonvaud-Funel, A. (2008) Growth and volatlie compound production by *Brettanomyces*/*Dekkera bruxellensis* in red wine. *Journal of Applied Microbiology*. **104**, 1577 - 1585.

Romano, A., Perello, M. C., Lonvaud-Funel, A., Silcard, G. & de Revel, G. (2009) Sensory and analytical re-evaluation of “Brett character”. *Food Chemistry*. **114**, 15 - 19.

Rossouw, M. & Marias, J. (2004) The phenolic composition of South African Pinotage, Shiraz and Cabernet Sauvignon wines. *South African Journal of Enology and Viticulture*. **25**, 94 - 104.

Saliba, A. J., Bullock, J. & Hardie, W. J. (2009) Consumer rejection threshold of 1,8-cineole (eucalyptol) in Australian red wine. *Food Quality and Preference*. **20**, 500 - 504.

Saurez, R., Saurez-Lepe, J. A., Morata, A. & Calderon, F. (2007) The production of ethylphenols in wine by yeasts of the genera *Brettanomyces* and *Dekkera*: A review. *Food Chemistry*. **102**, 10 - 21

Shinohara, T., Kubodera, S. & Yanagida, F. (2000) Distrubution of phenolic yeasts and production of phenolic off-flavours in wine fermentation. *Journal of Bioscience and Bioengineering*. **90**, 90 - 97.

Silva, P., Cardoso, H. & Gerós, H. (2004) Studies on the wine spoilage capacity of *Brettanomyces/Dekkera* spp. *American Journal of Enology and Viticulture*. **55**, 65 - 72.

Swiegers, J. H., Bartowsky, R. J., Henschke, P. A. & Pretorius, I. S. (2005) Yeast and bacteria modulation of wine aroma and flavour. In: *AWRI – Advances in wine science* (edited by R. J. Blair, M. E. Francis & I. S. Pretorius A. L.), Pp 159 - 187. Glen Osmond, Australia: Australian Wine Research Institute

Teixeira, M. I. V., Romão, M. V. S., Broze, M. R. & Boas, L. V. (2006) 2,4,6-Trichloroanisol: A consumer panel evaluation*. Ciência e Técnica Vitivinícola*. **2**, 53 - 65.

Thybo, A. K., Kühn, B. F. & Martens, H. (2003) Explaining Danish children's preferences for apples using instrumental, sensory and demographic/behavioural data. *Food Quality and Preference*. **15**, 53 - 63.

Tuorila, H., Monteleone, E. (2009) Sensory food science in the changing society: Oppurtunities, needs, and challenges. *Trends in Food Science & Technology*. **20**, 64 - 62.

Ugarte, P., Agosin, E., Bordeu, E. & Villalobos, J. I. (2005) Reduction of 4-ethylphenol and 4-ethylguaiacol concentration in red wines using reverse osmosis and adsorption. *American Journal of Enology and Viticulture*. **56**, 30 - 36.

Uscanga, M., Delia, M. & Strehaiano, P. (2000) Nutritional requirements of *Brettanomyces bruxellensis*: Growth and physiology in batch and chemostat cultures. *Canadian Journal of Microbiology*. **46**, 1046 - 1050.

Vichi, S., Romero, A., Tous, J., Tamames, E. L. & Buxaderas, S. (2009) Determination of volatile phenols in virgin olive oils and their sensory significance. *Journal of Chromatography A*. **1211**, 1 - 7.

Wirz, D. O., Heymann, H. & Bisson, L. F. (2004) Descriptive analysis of *Brettanomyces*-infected Cabernet Sauvignon wines. *American Journal of Enology and Viticulture*. **55**, 303A.

Wood, C., Siebert, T. E., Parker, M., Capone, D. L., Elsey, G. M., Pollnitz, A. P., Eggers, M., Meier, M., Vössing, T., Widder, S., Krammer, G., Sefton, M. A. & Herderich, M. J. (2008) From wine to pepper: Rotundone, an obscure sesquiterpene, is a potent spicy aroma compound. *Journal of Agricultural and Food Chemistry*. **56**, 3738 - 3744.

Wright, J. M. & Parle, J. N. (1974) *Brettanomyces* in the New Zealand wine industry. *New Zealand Journal of Agricultural Research*. **17**, 273 - 278.

Chapter 3: The determination of detection thresholds of eight *Brettanomyces*-related compounds in Pinotage red wine using two calculation methods

1 INTRODUCTION

Brett character is a wine spoilage defect which is associated with an unpleasant aroma which is commonly described as “horse-sweat” or “barnyard”. This defect most commonly occurs in red wine (Du Toit *et al.*, 2005). It is caused by spoilage by the yeast *Brettanomyces*, and its sporulating form, *Dekkera* (Loureiro & Malfeito-Ferreira, 2003). Sensory descriptors for wines with Brett character include rancid, band-aid™, soy, horsey, leather, tobacco and putrid (Wirz *et al.*, 2004). Brett character also masks inherent fruitiness in wines, as well as the varietal character (Licker *et al.*, 1999; Fugelsang & Zoecklein, 2003; Fariña *et al.*, 2007).

The two compounds most commonly associated with Brett character are the ethylphenols, 4-ethylphenol and 4-ethylguaiacol. These compounds were linked to the genus *Brettanomyces* in the early 1990’s (Chatonnet *et al*., 1992; 1993) and are generally considered “markers” for *Brettanomyces* spoilage. For this reason, these compounds are the subject of most routine tests for this microorganism in wine. A third ethylphenol, 4-ethylcatechol, was recently linked to *Brettanomyces* (Hesford *et al.,* 2004, Hesford & Schneider, 2004, Larcher *et al.*, 2008).

Isovaleric acid, a short-chain branched fatty acid, has also been linked to *Brettanomyces*, but not without controversy. Some authors have found a strong link between this compound and Brett character (Licker *et al.*, 1999; Fugelsang & Zoecklein, 2003), whereas other authors have found poor correlations between elevated levels of isovaleric acid and Brett character (Henske *et al.*, 2004). A recent study by Romano *et al.* (2009) found a strong link between the production of ethylphenols and high levels of both isovaleric acid and isobutyric acid. This implies the production of these two compounds by *Brettanomyces*, which may lead to their involvement in sensory interactions in terms of Brett character.

Two vinylphenols, 4-vinylphenol and 4-vinylguaiacol, are also associated with Brett character, as these compounds are both the precursors (Chatonnet *et al.*, 1992, 1993) and the breakdown products (Rayne & Eggers, 2007) of ethylphenols. The conversion of the vinylphenols to the ethylphenols is facilitated by vinylphenol reductase (Chatonnet *et al*., 1995), whereas the breakdown of the ethylphenols to vinylphenols is catalysed by *p*-cresol methylhydroxylase (PCMH) (Rayne & Eggers, 2007). However, these compounds are produced by numerous other wine micro-organisms in addition to *Brettanomyces*. *Brettanomyces* also produces varying amounts of acetic acid, which depends on the availability of oxygen to the organism (Du Toit *et al.*, 2005). Acetic acid, as in the case of the vinylphenols, can also be produced by several other wine micro-organisms.

A detection threshold can be defined as the lowest concentration at which a compound can be detected (but not necessarily recognised) by the senses. Although detection thresholds are usually either olfactory (detected by the sense of smell) or taste thresholds, the use of the sense of touch (for example for the determination of the detection threshold of a skin irritant) is

not impossible. Similarly, the recognition threshold of a compound is the level at which it can be recognised by the senses. In other words, the detection threshold is the level at which a difference can be detected, but the nature of the difference is not clear, whereas the recognition threshold is the level at which the difference can be recognised. This study focuses on the former. The detection thresholds for the above-mentioned compounds have been determined, but not necessarily in wine.

Chatonnet *et al.* (1992) determined the detection threshold of 4-ethylphenol and 4-ethylguaiacol in both water and model solution. These thresholds were defined as the minimum concentration under which 50% of 70 panellists failed to taste the difference from a control. The same authors also determined a "recovery threshold" in red wine by means of a triangular directional test, which was also defined as the level at which 50% of 70 panellists could correctly identify a sample containing the compound. These thresholds were 605 µg/L and 110 µg/L for 4-ethylphenol and 4-ethylguaiacol, respectively. The results found during this research coincidentally correspond to a significance level of less than 5% when evaluated using the statistical tables for difference testing (Roessler *et al*., 1987). However, using a chosen percentage of total panellists (50% in this case) as a basis instead of the statistical tables is still considered arbitrary and empirical (Lawless & Heymann, 1998). The methodology used by Chatonnet *et al*. (1992) is therefore still considered questionable.

The Australian Wine Research Institute (AWRI) undertook an investigation into the sensory attributes of Brett character in red wine, which included the determination of detection thresholds of 4-ethylphenol, 4-ethylguaiacol and 4-ethylcatechol (Curtin *et al*., 2008). These values were determined in three wines, namely a "neutral" wine, an "oaky" wine and a "green" wine. It was found that detection thresholds of these compounds are generally significantly higher in "oaky" wines than in neutral wines, and slightly higher in "green" wines than neutral wines. For example, the detection threshold of 4-ethylphenol was 368 µg/L in the neutral wine, 425 µg/L in the "green" wine and 569 µg/L in the "oaky" wine. The detection thresholds in the neutral wine were 158 and 774 µg/L for 4-ethylguaiacol and 4-ethylcatechol, respectively. The values for these two compounds in the "green" and "oaky" wines were not given. No details of the method used for the determination of detection thresholds were however included in the publication.

Apart from the investigations by the AWRI, the detection threshold of 4-ethylcatechol has been determined in two other reports. Hesford and Schneider (2004) defined this threshold as the level at which a difference could be tasted by their panel, and found the value to be 60 µg/L. However, no detail is given about the method used or the statistical basis for the test, and the validity of this value is therefore questionable. More recently, Larcher *et al.* (2008) used a duo-trio test and 5% significance levels from the Roessler tables (Roessler *et al.*, 1978) to determine a detection threshold for 4-ethylcatechol in white and red wine, and estimated this threshold in the range of 100 – 400 µg/L. However, their research does not specifically couple a detection

threshold to either red or white wine. It is interesting to note the vast differences between the three threshold values available for 4-ethylcatechol: 60 µg/L (Hesford & Schneider, 2004), 100 – 400 µg/L (Larcher *et al*., 2008) and 774 µg/L (Curtin *et al*., 2008). These discrepancies warrant further investigation into this compound's effect in wine.

Ferreira *et al.* (2000) determined the detection thresholds for isobutyric acid, isovaleric acid and 4-ethylguaiacol in a synthetic wine solution using a mixed triangle test. The synthetic wine solution contained 11% v/v ethanol, 7 g/L glycerin, 5 g/L tartaric acid and a pH of 3.4. The level at which 50% of the panellists could recognise the difference for the control were taken as the detection threshold value. The thresholds determined were 2300 µg/L, 33.4 µg/L and 33 µg/L for isobutyric acid, isovaleric acid and 4-ethylguaiacol respectively. However, in terms of the validity of this test, a similar argument as that applied to the research of Chatonnet *et al*. (1992) holds true.

Finally, the detection thresholds for acetic acid and 4-vinylguaiacol were determined by Guth (1997) in a water and ethanol mixture where the ratio was 9:1, through a method that is only described as "nasal comparison". A detection threshold that of was 2 000 000 µg/L, or 0.2 g/L was found for acetic acid. The result found for 4-vinylguaiacol was 40 µg/L. These results are questionable, as no accepted sensory or statistical method was applied.

The American Standard Test Manual (ASTM) E679 method has been used to determine detection thresholds by several other authors, both in wine and in other media. Examples in wine include the determination of the detection threshold for rotundone (Wood *et al*., 2008), oak lactones (Brown *et al.*, 2006) and diacetyl (Martineau *et al*., 1995). This method involves presenting judges with 3-alternative forced choice tests in increasing concentration until the odd sample has been correctly identified in two consecutive cases. This gives rise to a best estimate threshold (BET) value, which is the geometric mean of the last incorrectly identified sample and the first correctly identified sample. The test is performed over a set range of concentrations, which is determined beforehand by means of sensory testing. However, as the test evaluates a predetermined concentration range, the data produced are both top and bottom truncated. This means that the range in which the test functions has both a top and a bottom limit, which can be problematic if the detection threshold falls close to these limits.

The determination of odour thresholds uses the combined sensory response of a selected group of trained individuals, called panellists. However, the correct identification of an odorant at very low concentration levels in a specific wine can pose several challenges. These include the varying sensitivity of panel members (a factor affected by physiological differences or professional experience), tiredness of sense organs, temporal persistence of a characteristic aroma, as well as synergistic effects of different compounds on the sensory character of a wine (Le Berre *et al.*, 2007). Furthermore, specific compounds such as alcohol or other aromatic wine compounds – especially fruity and woody odourants – can have a significant effect on the perception of Brett character in different media (Lawless, 1999; Le Berre *et al.,* 2007; Escudero

et al., 2007). This has been demonstrated for Brett character by the research of Curtin *et al*. (2008). However, it has been shown that the use of training has a positive effect on the accuracy of data determined from triangle tests (Dacremont & Sauvageot, 1997).

The aim of this study was to determine the detection thresholds in Pinotage red wine for eight compounds, namely 4-ethylphenol, 4-ethylguaiacol, 4-ethylcatechol, isovaleric acid, isobutyric acid, 4-vinylphenol, 4-vinylguaiacol and acetic acid. This served several purposes. Firstly, none of the detection thresholds of these compounds have been determined in Pinotage red wine to date. Pinotage, which was bred in South Africa, forms an important part of South African red wine's portfolio and the determination of these values thus has a potential benefit for the South African wine industry. Furthermore, as described earlier, for some of these compounds, the only literature values available are for detection in either water or model solution (Guth, 1997; Ferreira *et al.*, 2000; Curtin *et al.*, 2005), and it has been shown that detection thresholds found in model solutions are not truly comparable to those determined in wine (Le Berre *et al.,* 2007). Wine style and type has also generally been found to have an effect on detection threshold (Martineau *et al*., 1995; Brown *et al.*, 2006). Finally, as the sensory effects of these compounds were tested in greater detail in subsequent studies (see Chapter 4); it was of utmost importance to initially determine the detection thresholds of these compounds in Pinotage.

2 MATERIALS AND METHODS

2.1 Samples

Three hundred litres of Pinotage red wine was supplied by a local producer of wines (Distell Group Ltd, Stellenbosch, South Africa) during the course of 2008 and was bottled manually at the Department of Viticulture and Oenology, Stellenbosch University, South Africa. The wine was made using standard red wine making practices and completely underwent both alcoholic and malolactic fermentation. The wine had a pH of 3.7, and an alcohol concentration of 12.9 %. The wine had not been wooded prior to bottling, and had an aroma profile that was dominated by fruit.

After bottling, samples were taken to determine the levels of the compounds investigated in this study in the wine. Analysis of 4-ethylphenol, 4-ethylguaiacol, 4-vinylphenol, 4-vinylguaiacol, isovaleric acid, isobutyric acid and acetic acid were performed using GC-MS. The analysis for 4-ethylcatechol was performed using HPLC-MS. All these analyses were conducted by an accredited wine analysis laboratory (Quantum Laboratories, South Africa).

The wine used in this study was found to contain 6 µg/L 4-ethylphenol, 4 µg/L 4-ethylguaiacol and 0 µg/L 4-ethylcatechol. The wine was therefore considered to be free of

Brettanomyces spoilage. However, the wine contained 370 µg/L 4-vinylphenol, and 29 µg/L 4-vinylguaiacol. Both these values are rather high. The isovaleric acid concentration was 168 µg/L and the isobutyric acid concentration 68 µg/L. Both these values are well below those typically found in red wine (Francis & Newton, 2005). Finally, the acetic acid concentration was 0.20 g/L.

Table 3.1. Concentration ranges used for spiking wines during the determination of detection thresholds of *Brettanomyces* related compounds in Pinotage wine.

Compound	MF[a]	Concentration used							
		1	2	3	4	5	6	7	8
4-ethylphenol (µ/L)	1.4	98	137.2	192	268	376	527	737	1033
4-ethylguaiacol (µ/L)	1.4	71	99	139	195	273	382	535	748
4-ethylcatechol (µ/L)	1.4	99	139	194	272	380	532	745	1044
Isovaleric acid (µ/L)	1.7	12	20.4	34	58	100	170	289	492
Isobutyric acid (µ/L)	1.7	260	442	751	1277	2171	3691	6275	10668
4-vinylphenol (µ/L)	1.25	6.4	8	10	12.5	15.6	19.5	24.4	30.5
4-vinylguaiacol (µ/L)	1.3	5	6.5	8.45	10.9	14.28	18.56	24.1	31.3
Acetic acid (g/L)	1.5	0.1	0.15	0.225	0.337	0.506	0.759	1.139	1.708

[a]Multiplication factor: factor used for multiplication between different levels

Solutions of 10 mg/mL, 1 mg/mL, 100 µg/mL and 10 µg/mL of the eight compounds (4-ethylphenol, 4-ethylguaiacol, 4-ethylcatechol, 4-vinylphenol, 4-vinylguaiacol, isovaleric acid, isobutyric acid and acetic acid) (Aldrich, South Africa) were prepared in 99.5% ethanol (Merck Chemicals, South Africa). These solutions were used to "spike" the wines with the concentrations listed in Table 3.1 so that the final concentrations in the wines correlated to those listed in Table 3.2. Concentration ranges for all eight of the compounds involved were sourced from literature, and were originally evaluated by a panel of three to four wine industry consultants (Thales South Africa, IWBT, Stellenbosch University) who are considered experts in the field of "Brett character." These concentration ranges consisted of 11 concentrations, with the literature maximum set at approximately level 8, and the literature detection threshold set at approximately level 5. These samples were analysed by means of sensory analysis by consensus using the panel of consultants. The final concentrations used in this study were decided upon during these analyses, and are shown in Table 3.2.

2.2 Determination of detection threshold levels

The determination of the detection threshold levels was carried using a method based on the standard method of the American Standard Test Manual (ASTM E 679 – 04). This method is the standard practice for the determination of odour and taste thresholds by a forced choice ascending concentration series method of limits. The test is defined as a three-alternative forced

choice (3-AFC) test, and involves presenting judges with sets of three samples, of which one contains the compound in question. Judges are then instructed to identify the odd sample in each set. Sample sets are presented in ascending order of concentration. This ASTM method prescribes that the test should be terminated as soon as a judge made two consecutive correct choices, in other words, as soon as the "spiked" sample was correctly identified in two consecutive concentrations. A more detailed description of the test, the training involved, the modifications to the test and the data analysis method used follow in the appropriate sections.

Table 3.2. Final concentration ranges used in the determination of detection thresholds of *Brettanomyces* related compounds in Pinotage wine.

Compound	MF[a]	Concentration used							
		1	2	3	4	5	6	7	8
4-ethylphenol (µ/L)	1.4	104	143.2	198	274	382	533	743	1039
4-ethylguaiacol (µ/L)	1.4	75	103	143	199	277	386	539	752
4-ethylcatechol (µ/L)	1.4	99	139	194	272	380	532	745	1044
Isovaleric acid (µ/L)	1.7	180	188.4	202	226	268	338	457	660
Isobutyric acid (µ/L)	1.7	328	510	819	1345	2239	3759	6343	10736
4-vinylphenol (µ/L)	1.25	376	378	380	382.5	385.6	389.5	394.4	400.5
4-vinylguaiacol (µ/L)	1.3	34	35.5	37.45	39.9	43.28	47.56	53.1	60.3
Acetic acid (g/L)	1.5	0.3	0.35	0.425	0.537	0.706	0.959	1.339	1.908

[a]Multiplication factor: factor used for multiplication between different levels

2.2.1 Subjects and training

A trained panel was used for the determination of detection thresholds of the eight compounds (4-ethylphenol, 4-ethylguaiacol, 4-ethylcatechol, 4-vinylphenol, isovaleric acid, isobutyric acid and acetic acid) in the Pinotage red wine described above. The panel consisted of 10 individuals who had experience participating in sensory tests. A portion of the panel had previously participated in an extensive study for the determination of the detection thresholds of cork-taint related compounds, and therefore was experienced in sensory analysis of wine, as well as the use of the triangle test. However, at the onset of the project the panel had no or limited experience in detecting Brett character in wine and the ten judges were thus trained extensively in the detection of the above-mentioned compounds in Pinotage. The ASTM test is a 3-AFC test, which requires that the differences between samples should be known prior to the analysis (O'Mahony, 1995). Training of judges was therefore motivated by the fact that the aromas associated with the different compounds are extremely difficult to communicate verbally.

The panel was trained in two phases. In *Phase 1* each judge received a control sample containing only the base wine, as well as three wine samples and one water sample spiked with the compound in question. The first wine sample contained the lowest level that were be used in

the test (level 1, see Table 3.1). The second wine sample contained the compound in question at the level closest to its assumed detection threshold (usually level 4 or 5, Table 3.1). The third wine sample contained the compound in question at the highest level that will be tested (level 8, see Table 3.1). The water sample, which was used as a reference sample, contained the compound at the highest level that will be tested (level 8, see Table 3.1). These samples were used to characterise the aromas of the specific compounds, and to familiarise the judges with the particular aroma associated with each compound. The judges received the samples in a "round-table" situation where the differences between the wines were discussed and descriptors identifying the differences between the samples were generated. The identification of difference, as well as the generation of descriptors, was an essential part of performing the test, as the difference between samples should be known for the performance of a true 3-AFC test (O'Mahony, 1995).

In *Phase 2* of training each judge received eight sets of samples, each set containing three samples. In each set, two of the three samples contained only the base wine (untainted wine) and a third sample in every set contained the base wine plus the added compound. The concentration increased with a constant factor from set 1 through to set 8 as illustrated in Table 3.1. The sets were presented in an order of ascending concentration and the samples within each set were presented in a randomised order. The sample volume was 20 mL and all the samples were served in ISO wine tasting glasses at 20 ±1°C. Each sample was numbered with a random three-digit code and was covered with a tight-fitting lid to prevent the aroma from escaping and contaminating the laboratory environment.

The judges were instructed to smell the headspace of the samples in each set, i.e. in the order presented and then to indicate the *odd* or tainted sample. Each judge was required to rest for a total of 2 min between every set and 5 min between the fourth and fifth set which was regarded as the *half-way mark* for this procedure. The latter was to cancel strong carry-over effects and to minimise tiredness of the sense organs. This procedure was repeated until consensus was reached on the odd sample within each set. *Phase 2* of training was performed for each of the eight compounds used in this study.

2.2.2 Determination of detection thresholds

The detection thresholds of the different compounds were determined by the panel as described in *Phase* 2 of the training. The panel determined the detection levels of all eight compounds (4-ethylphenol, 4-ethylguaiacol, 4-ethylcatechol, 4-vinylphenol, isovaleric acid, isobutyric acid and acetic acid) in Pinotage red wine. The final analyses were conducted by 10 trained assessors in booths with standard artificial daylight lighting and temperature control at 20°C ±1°C. Communication was not allowed between the judges for the duration of the test. Although the ASTM stipulates terminating the test after a judge detected a compound, the judges received all

eight sample sets, and their responses were only assessed after the test, as the responses of the judges could not be assessed during the test. Each detection threshold test consisted of four replicates of the ASTM E679 - 04 test.

2.2.3 Analysis of data

An example of the results obtained from this test is shown in Table 3.3. The ASTM E679–04 method prescribes that "detection" is defined as correctly identifying the spiked sample from the two unspiked samples in two consecutive sample sets. "0" indicates incorrect identification of the spiked sample, and "+" indicates correct identification of the spiked sample. The levels marked with the superscript "a", are the levels at which the judges detected the compound according to the ASTM method. All data regarding further sample sets are ignored. From these results, the detection thresholds are calculated in two steps.

Table 3.3. Example of results obtained from ASTM method. Note that the concentrations are hypothetical. "0" indicates incorrect identification of the spiked sample, and "+" indicates correct identification of the spiked sample.

Judge	Level								BET[b, c]
	1	2	3	4	5	6	7	8	
1	0	0	0	0[a]	+	+	+	+	$(c_4 \times c_5)^{1/2}$
2	0	0	+	0	0[a]	+	+	+	$(c_5 \times c_6)^{1/2}$
3	0	+	0[a]	+	+	+	+	+	$(c_3 \times c_4)^{1/2}$
4	0	0	0[a]	+	+	+	+	+	$(c_3 \times c_4)^{1/2}$
5	0	0[a]	+	+	0	+	+	+	$(c_2 \times c_3)^{1/2}$
6	+	+	+	+	0	0	0	0	$(c_0 \times c_1)^{1/2}$
7	0	+	0	0	+	0	+	0[a]	$(c_8 \times c_9)^{1/2}$
8	0[a]	+	+	0	0	+	+	+	$(c_1 \times c_2)^{1/2}$

[a] Level which is considered the first instance of "detection". In the case of Judge 6, the level that would have preceded level 1 is considered to be the first instance of "detection". [b] BET = Best Estimate Threshold value obtained in specific instance of the test. [c] c designates the value of the actual concentration used in the test for that specific level.

Firstly, the best estimate threshold (BET) is determined per judge per sample set. This is calculated using equation (1).

1) $BET = \sqrt{c_1 \times c_2}$

Where:

BET = Best estimate threshold

c_1 = last missed concentration

c_2 = next concentration after c_1

Simply put, the BET values are the geometric mean of the last concentration that the judge could not detect, and the first concentration that was considered as “detected”. The geometric mean is used in order to compensate for the fact that the concentration values used fall on a logarithmic scale.

Once the BET values for all judges and replications were calculated, the detection thresholds were calculated either using the median or the prescribed ASTM E679–04 method. The calculation used for the ASTM E679–04 method is show in equation (2).

2) $$DT = \sqrt[n]{\prod_{1}^{n} BET}$$

Where:

DT = detection threshold

n = number of repetitions (Judge x Replications)

In other words, the detection threshold is calculated as the geometric mean of the BET values over all judges and replicates. A simple method of calculating this is by finding the mean of the logarithms of the BET values, and then finding the antilog of the mean. When the median was used, the median of all the logarithmic values was found, and the antilog of these values was said to be the detection threshold.

A 95% confidence interval was calculated for both the ASTM method and for the median. The confidence interval of a value is an interval in which it can be said that the estimate of the value falls with a quantitative degree of statistical certainty. The range of the interval can be considered as a measure of accuracy for a statistical method: a method with a smaller range is considered to be more accurate (Neyman, 1937). The confidence interval of the median was calculated using the method described by (Snedecor & Cochran, 1967) by finding the order statistics with the following numbers:

3) $$UL_{os} = \frac{(n+1)}{2} + \frac{z\sqrt{n}}{2}$$

4) $$LL_{os} = \frac{(n+1)}{2} - \frac{z\sqrt{n}}{2}$$

Where:

UL_{os} = Order statistics for upper confidence limit

LL_{os} = Order statistics for lower confidence limit

z = normal deviate corresponding to the desired probability (in this case 0.05)

n = number of responses

Note that the order statistic does not refer to the actual value of the confidence limit, but to the ranking of a number that corresponds to this confidence limit.

3 RESULTS AND DISCUSSION

3.1 Comparison between different calculation methods

The results obtained from the two different calculation methods are compared in Table 3.4. Note that base wine concentrations are not taken into account in these results. These results are shown visually in Figures 3.1 to 3.8.

Table 3.4. Comparison of two calculation methods for determination of detection thresholds. In this table, LL designates the lower confidence limit of each value, and UL designates the upper confidence limit. Note that base wine concentrations are not taken into account in these results.

Compound	ASTM LL	ASTM Value	ASTM UL	Median LL	Median Value	Median UL
4-ethylphenol (µ/L)	174	221	281	162	195	250
4-ethylguiacol (µ/L)	87	107	130	60	84	117
4-ethylcatechol (µ/L)	316	442	618	229	385	881
Isovaleric acid (µ/L)	44	72	117	26	44	222
Isobutyric acid (µ/L)	1132	1756	2726	576	1666	4813
4-vinylphenol (µ/L)	10	14	20	10	11	26
4-vinylguaiacol (µ/L)	9	12	16	7	11	28
Acetic acid (g/L)	0.165	0.204	0.252	0.122	0.108	0.298

Some general trends can be observed from all these datasets. Firstly, the median gives a lower estimation of detection threshold in all cases, but in many cases the estimation given by the median falls between the same two levels than the estimation given by the ASTM method. In some cases, (for example acetic acid, Figure 3.8) the difference between the median and its lower level is smaller than the difference between the ASTM and its lower level. However, in almost all of the datasets, the difference between the median and its upper limit (up to 5 levels) is significantly larger than the difference between the ASTM and its upper limit (usually 1 level).

Although the use of the median generally gives a larger confidence interval than the ASTM method and may therefore be considered to be less accurate (Neyman, 1937), the median and its confidence limits give a much better snapshot of the performance or abilities of the sensory panel, whereas the ASTM method may be an oversimplified method of calculating detection thresholds.

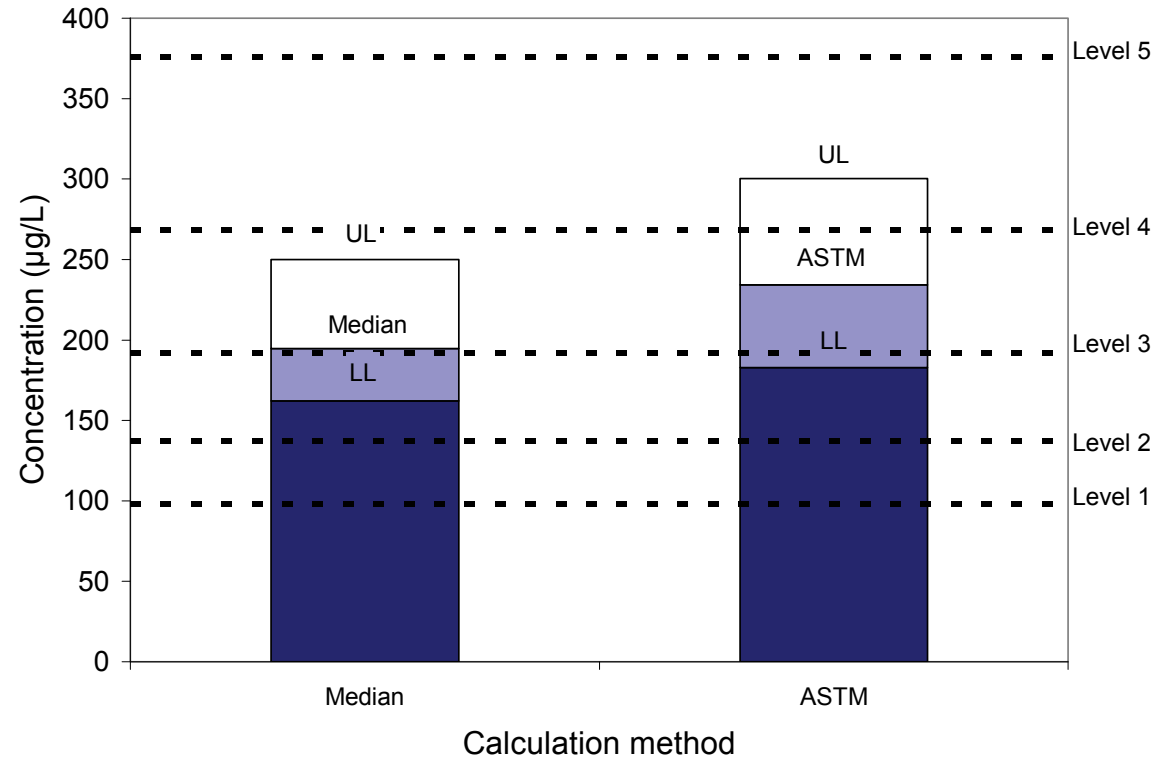


Figure 3.1. Effect of calculation methods on detection threshold of 4-ethylphenol. LL designates the lower confidence limit of each value, and UL the upper confidence limit.

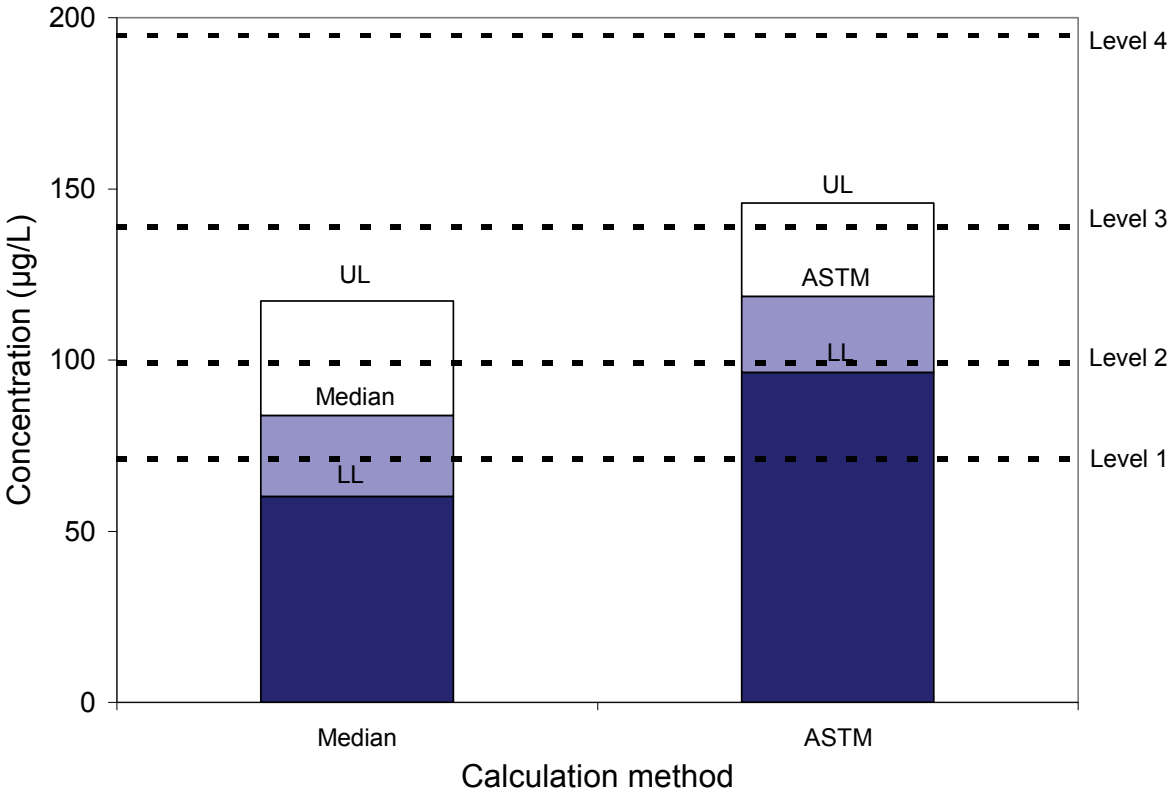


Figure 3.2. Effect of calculation method on detection threshold of 4-ethylguaiacol. LL designates the lower confidence limit of each value, and UL the upper confidence limit.

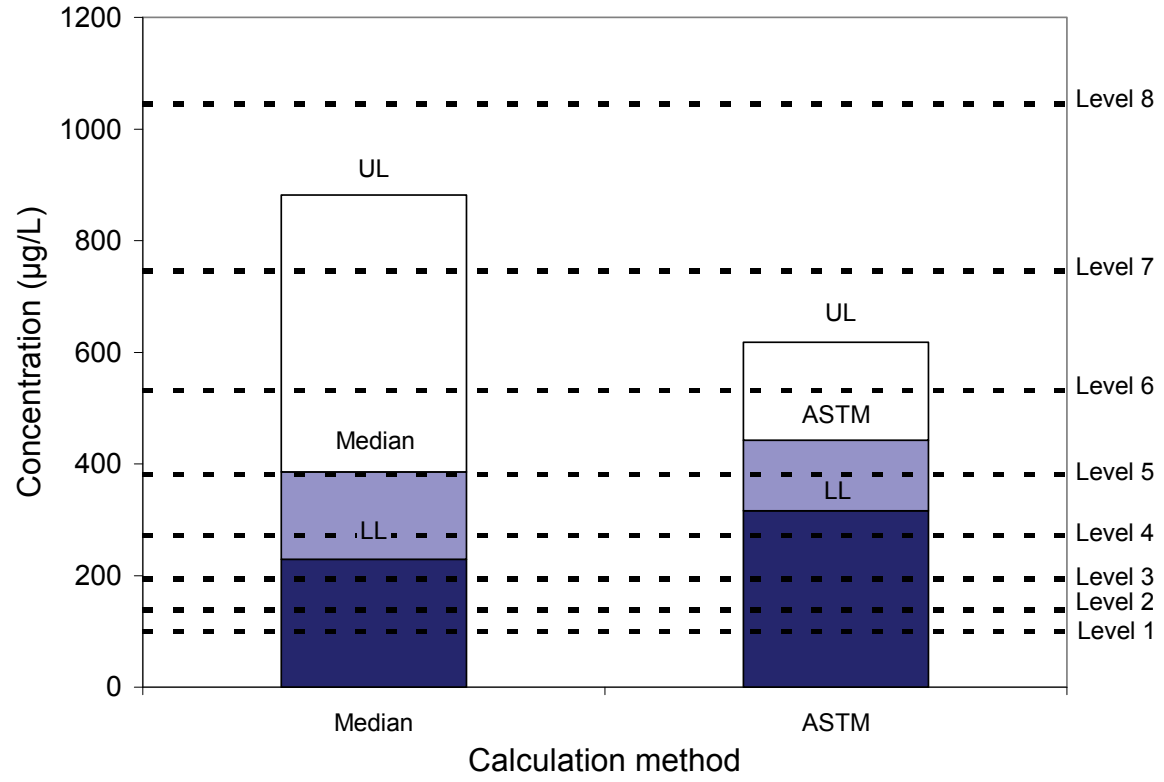


Figure 3.3. Effect of calculation method on detection threshold of 4-ethylcatechol. LL designates the lower confidence limit of each value, and UL the upper confidence limit.

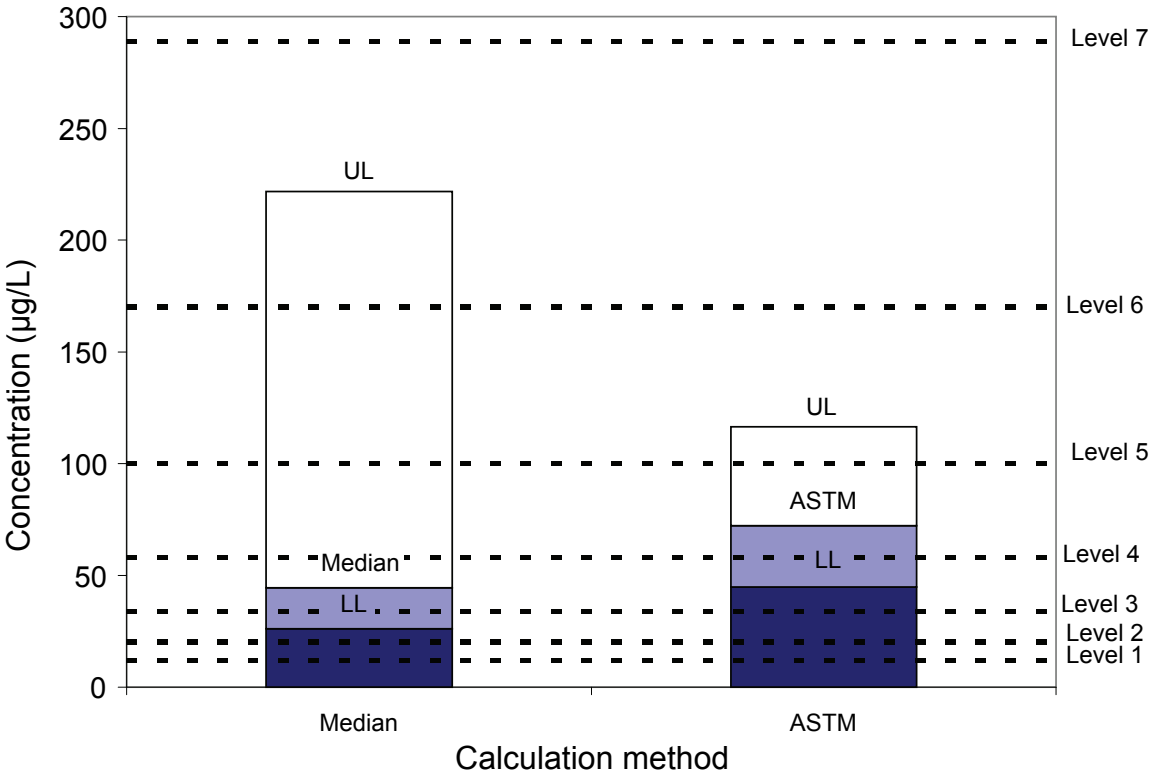


Figure 3.4. Effect of calculation method on detection threshold of isovaleric acid. LL designates the lower confidence limit of each value, and UL the upper confidence limit.

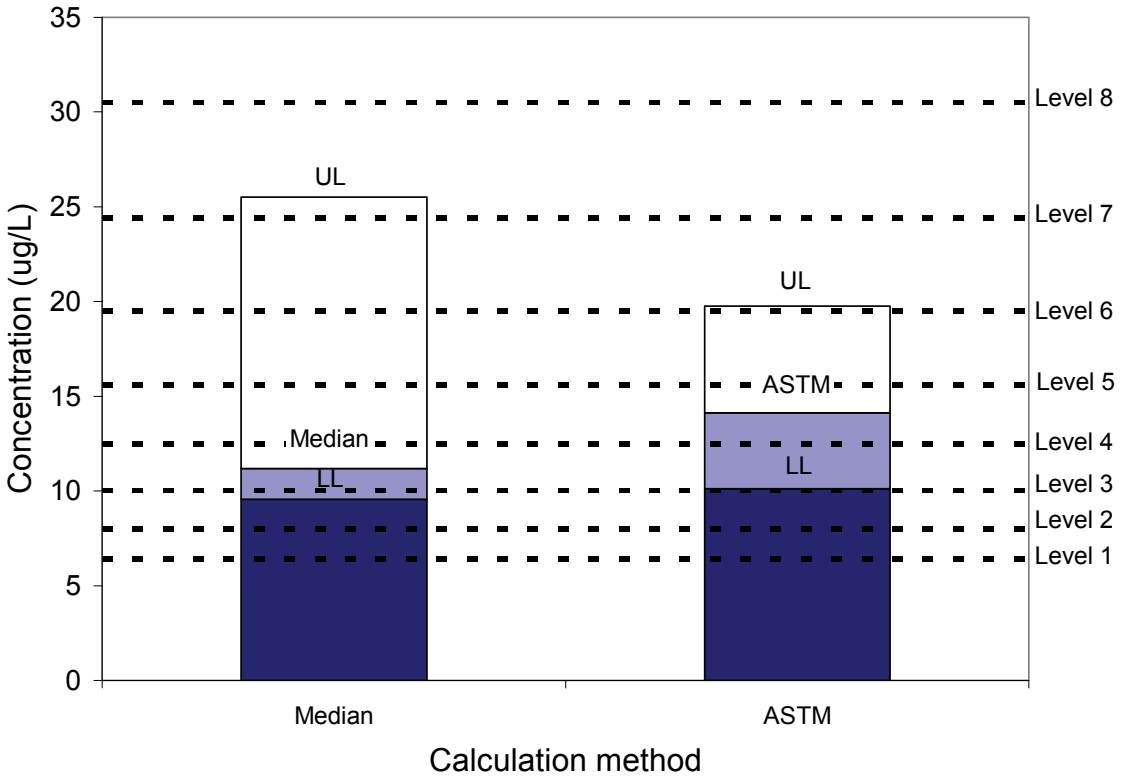


Figure 3.5. Effect of calculation method on detection threshold of 4-vinylphenol. LL designates the lower confidence limit of each value, and UL the upper confidence limit.

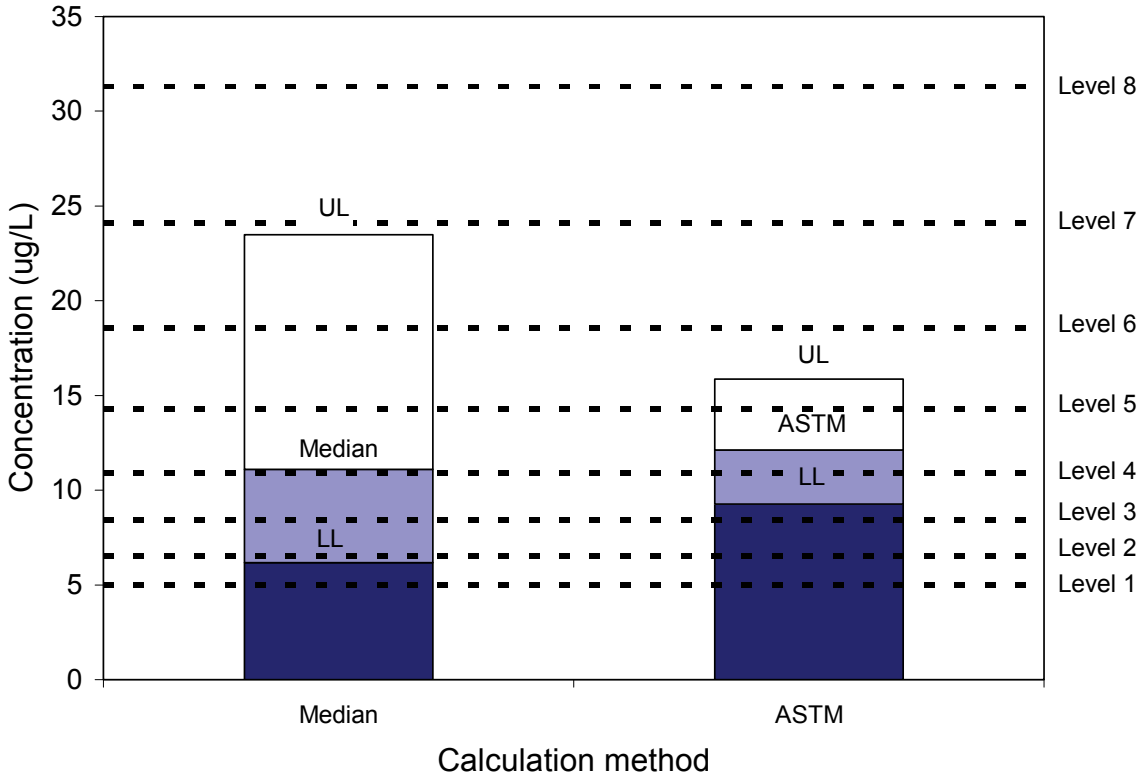


Figure 3.6. Effect of calculation method on detection threshold of 4-vinylguaiacol. LL designates the lower confidence limit of each value, and UL the upper confidence limit.

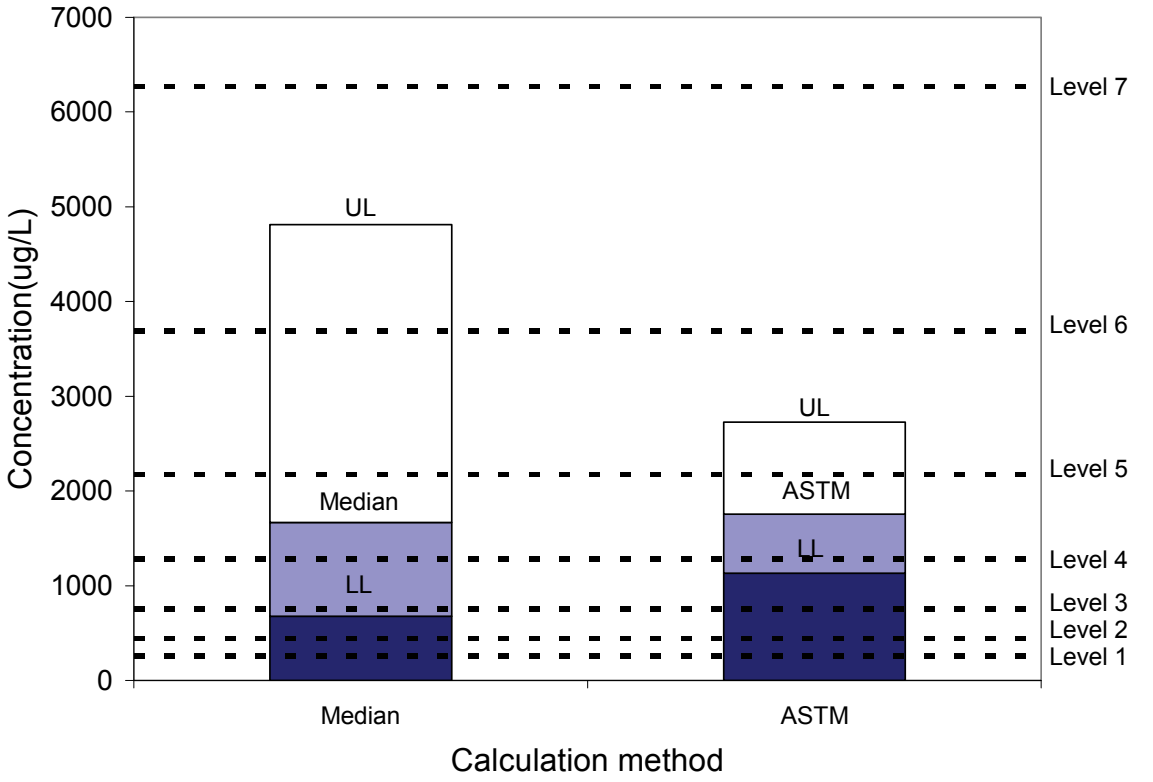


Figure 3.7. Effect of calculation method on detection threshold of isobutyric acid. LL designates the lower confidence limit of each value, and UL the upper confidence limit.

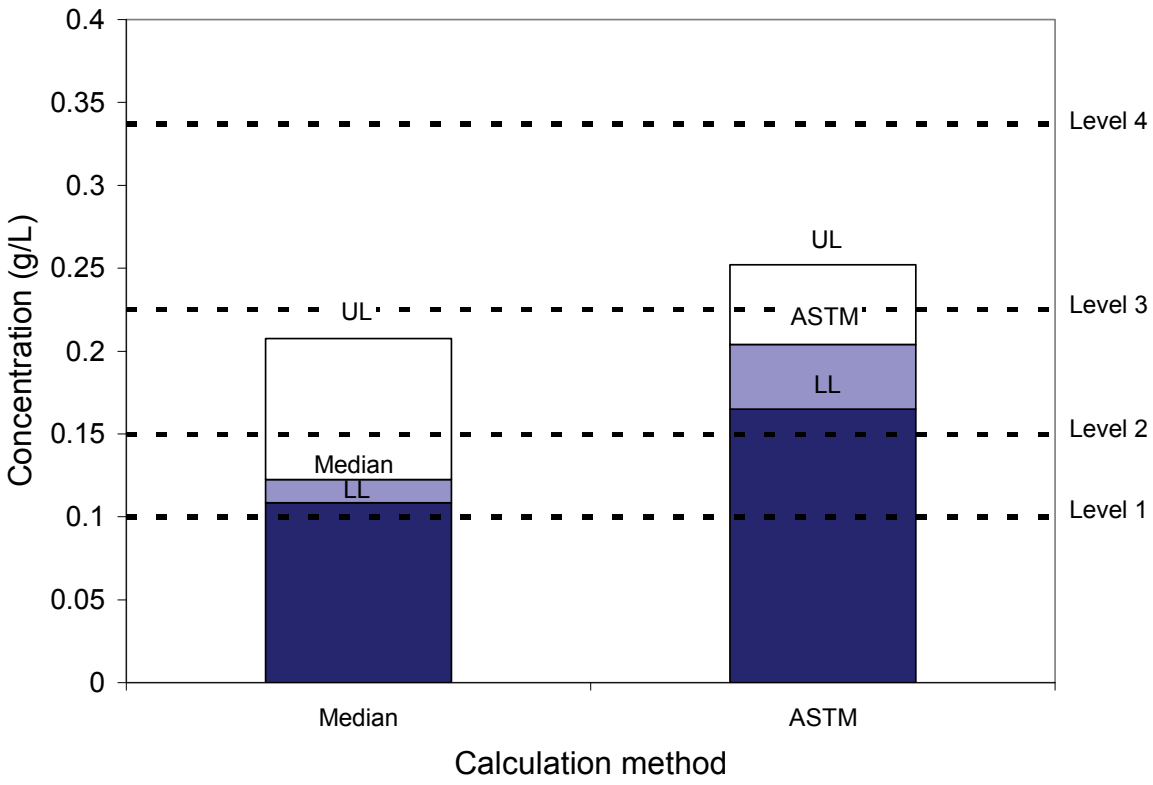


Figure 3.8. Effect of calculation method on the detection threshold of acetic acid. LL designates the lower confidence limit of each value, and UL the upper confidence limit.

The median gives an indication where most of the instance of detection occurred, whereas the ASTM method produces an “average” result. This is particularly important as the data being analysed is both top and bottom truncated and can therefore only fall into a predetermined range. This means that detecting a compound at the lowest level may indicate that the individual could have detected this compound below the lowest level presented to judges. However, this effect is “averaged out” when the ASTM method is used.

The median also gives a good indication of the general ability of each compound to be detected by individuals. A median with an upper limit that is much higher than the median indicates that although most individuals detected the compound at a certain level, it could only be detected at higher concentrations in several other instances. This gives an indication of the distribution of the variability in detection ability of a specific compound between judges. For example, the lower detection limit and the detection limit of 4-vinylphenol (Figure 3.5) both lie between level 3 and 4, whereas the upper detection limit lies between level 7 and 8. This indicates that most individuals were able to detect this compound at between level 3 and 4; some individuals were only able to detect it at much higher levels.

The differences in results obtained by these two calculation methods raises the question of the true definition of the term “detection threshold”. In ASTM E679-04, detection threshold is defined as “the lowest concentration of a substance in a medium relating to the lowest physical intensity at which a stimulus is detected as determined by the best-estimate criterion”. This implies that the detection threshold of a compound should be set at the lowest level that the compound can be detected by most individuals, regardless of whether or not other individuals are less able to detect this compound at those concentrations. If this is taken into account, the median appears to be a more suitable method for the calculation of detection threshold, as the result obtained from this calculation method is not as affected by the variation between judges.

An alternative method of compensating for the varied abilities of judges may lie in identifying judges that perform poorly or inconsistently, and subsequently omitting their observations from the data set. Looking at the dataset as a whole may give a much better indication of whether or not a judge truly detected the compound or if the two consecutive correct indications required for “detection” occurred simply by chance. It is recommended that future studies focus on the diagnostics of detection-threshold type data and that a diagnostic test be developed for sets of detection threshold data. Such a test should not only take into consideration the probability correctly identifying the “spiked” sample by chance, but also the probability of it occurring twice, three times and so forth, and the statistical implications of incorrect indications once two consecutive correct identifications have occurred.

A further statistical issue that could not be addressed in this study but deserves future attention is the type of detection threshold test used. The ASTM E679-04 is defined as using a three-alternative forced choice (3-AFC) test, which it loosely describes as “a set consisting of one test sample and two blank samples”. However, the definition of the 3-AFC test is not quite

as simple as the standard makes it out to be. O'Mahony (1995) defines a 3-AFC test as a test where the sensory differences between the samples are known to the judges performing the test. He also states that the probability of a judge correctly identifying an odd sample in a 3-AFC test is significantly higher than in a simple triangle test where the difference is not known. This difference in performance and probability is of a sufficient magnitude to give different outcomes should binomial proportional statistics like the tables developed by Roessler *et al*., (1978) be applied. This means that in some cases, the application of a true 3-AFC test would have the outcome that the samples are significantly different according to the Roessler tables, but applying a normal triangle test (where the difference is not known) would have the outcome that the samples are not significantly different.

The implications for this study are that although the principles for performing a 3-AFC test were followed, severe difficulties were experienced in defining the difference in sensory profile caused by some of the compounds. Two specific examples were 4-ethylcatechol and isobutyric acid as for both these compounds a difference in the aroma profile of the wine could be perceived, but the difference was not easily identifiable. In the case of other compounds, like 4-ethylguaiacol, the difference in sensory profile was immediately identified by all the judges. The implication of this is that the statistical principles on which ASTM E679-04 are based may not be equally appropriate for all instances that this test may be used.

3.2 Comparison to literature

The results obtained with both calculation methods are compared with their literature values in Table 3.5. The "total" results (the detection thresholds obtained plus the concentration present in the base wine) are shown in Table 3.6.

As can be seen in Table 3.5, the detection thresholds found using the median method during this study are generally lower than those reported in literature. However, when the levels of these compounds present in the wine prior to addition is taken into consideration (Table 3.6), detection threshold are lower in some cases (4-ethylphenol and isobutyric acid), comparable in some cases (4-vinylguaiacol and 4-ethylguaiacol) and higher in other cases.

The values obtained for 4-vinylguaiacol and 4-ethylguaiacol (Table 3.6) are similar to those obtained from literature (Chatonnet *et al*., 1992; Guth, 1997). However, the median obtained for 4-ethylguaiacol is significantly lower than that obtained through the ASTM. From this it could be concluded that there existed a difference in sensitivity between judges in terms of the perception of 4-ethylguaiacol. This is in line with the findings of Laska & Hudson (1991) and Curtin *et al*. (2008).

Table 3.5. Comparison of detection thresholds obtained with two calculation methods to literature.

Compound	Median	ASTM	Literature value	Reference
4-ethylphenol (µg/L)	195	221	605[a]	Chatonnet *et al.*, 1993
Range:	162 - 250	174 - 281		
4-ethylguaiacol (µg/L)	84	107	110[a]	Chatonnet *et al.*, 1993
Range:	60 - 117	87 - 130		
4-ethylcatechol (µg/L)	385	442	60[a]	Hesford & Schneider, 2004
Range:	229 - 881	316 - 618		
Isovaleric acid (µg/L)	44	72	33[b]	Ferreira *et al.,* 2000
Range:	26 - 222	44 - 117		
Isobutyric acid (µg/L)	1666	1756	2300[b]	Ferreira *et al.,* 2000
Range:	676 - 4813	1132 - 2726		
4-vinylphenol (µg/L)	11	14	180[b]	Culleré *et al*., 2003
Range:	10 - 26	10 - 20		
4-vinylguaiacol (µg/L)	11	12	40[b]	Guth, 1997
Range:	7 - 28	9 - 16		
Acetic acid (g/L)	0.122	0.204	0.20[b]	Guth, 1997
Range:	0.108 – 0.207	0.165 - 0.252		

[a] Determined in red wine. [b] Determined in model solution.

The total detection thresholds (Table 3.6) of isovaleric acid determined using both methods were both significantly higher than the detection threshold obtained from literature (Ferreira *et al*., 2000). This is a direct result of the fact that the wine used in this study contained a fair amount of this compound. The higher detection threshold can also be ascribed to the fact that the literature detection threshold was determined in model solution, and that it is generally more difficult to detect odorants in real wines than in model solution (Le Berre *et al.,* 2007). The higher detection thresholds for 4-vinylphenol and acetic acid found in this study compared to literature (Guth, 1997; Culleré *et al*., 2003) are further evidence of this phenomenon.

Both detection thresholds (Table 3.6) determined for 4-ethylphenol were substantially lower than that reported in literature. This can be ascribed to the fact that the wine we used was not strongly wooded, and 4-ethylphenol falls in the same semantic category as woody odourants (Escudero *et al*., 2007). This means that for wooded wines, the wood character suppresses some of the aroma character of 4-ethylphenol, making it more difficult to detect. This is in agreement with the findings of Curtin *et al*. (2008). Conversely, 4-ethylphenol is easier to detect in a wine with a lower level of woodiness, leading to a lower detection threshold in this study.

Another relevant sensory aspect of the wine used is the fact that it was a very fruity wine, which may also affect the detection thresholds of certain compounds. Isobutyric acid was described by the panel as having a fruity/floral character, and it is possible that the lower detection threshold found for isobutyric acid is a result of the amplifying effect that it may have on the already high fruitiness of the wine, making it easier to detect at lower concentrations.

Guadagni *et al*. (1963) postulated a theory of additive effects between odour compounds, which stated that detection thresholds of compounds are lower when they are present in solution with other compounds. Although it has been found (Grosch, 2001) that this theory does not hold true in all cases, synergism has been found between specific odour pairs (Laing, 1988; Laska & Hudson, 1991). The effect exhibited by isobutyric acid in this study may be an example of such a type of synergism.

Table 3.6. Total detection thresholds (detection thresholds obtained plus base wine concentration).

Compound	Median	ASTM	Literature value	Reference
4-ethylphenol (µg/L)	201	221	605[a]	Chatonnet *et al.*, 1993
Range:	168 - 256	180 – 287		
4-ethylguaiacol (µg/L)	84	111	110[a]	Chatonnet *et al.*, 1993
Range:	64 - 121	91 - 134		
4-ethylcatechol (µg/L)	385	442	60[a]	Hesford & Schneider, 2004
Range:	229 - 881	316 - 618		
Isovaleric acid (µg/L)	214	242	33[b]	Ferreira *et al.,* 2000
Range:	196 - 392	214 - 287		
Isobutyric acid (µg/L)	1735	1825	2300[b]	Ferreira *et al.,* 2000
Range:	676 - 4813	1132 - 2726		
4-vinylphenol (µg/L)	381	384	180[b]	Culleré *et al*., 2003
Range:	380 - 396	380 - 390		
4-vinylguaiacol (µg/L)	40	41	40[b]	Guth, 1997
Range:	36 - 57	38 - 45		
Acetic acid (g/L)	0.322	0.404	0.20[b]	Guth, 1997
Range:	0.308 – 0.407	0.365 - 0.452		

[a] Determined in red wine. [b] Determined in model solution.

The highest discrepancy between threshold values obtained here and reported in literature was observed for 4-ethylcatechol. This may be due to the fact that Hesford and Schneider (2004) did not use an accepted methodology for the determination of the detection threshold of 4-ethylcatechol, as their article gave no details for the method that they employed for the determination of this detection threshold. A more recent study, by Larcher *et al.* (2008) also struggled to detect 4-ethylcatechol at the concentration found by Hesford and Schneider (2004), and placed the detection threshold for 4-ethylcatechol in the range of 100 – 400 µg/L, which is more in line with our observations. However, the value that this research found was much lower than the value of 774 µg/L found by Curtin *et al*. (2008). This may be due to the test method used. As Curtin *et al*. (2008) used Cabernet Sauvignon wine in their study, which could be less prone to being affected by 4-ethylcatechol than a fruity, unwooded Pinotage.

4 CONCLUSIONS

In this study, the detection thresholds for eight different compounds associated with *Brettanomyces* were determined. Two data analysis methods were compared. It was found that although using the median gives a lower detection threshold and a larger confidence interval, it gives a better indication of overall panel performance. Some discrepancies were found between the literature values and those determined experimentally. Most of these discrepancies could be clarified by an investigation into the methods employed to establish these threshold values, as well as the aroma profiles of the wine used. These discrepancies, as well as the reasons for the differences, justified the determination of these detection thresholds before proceeding to further sensory studies.

5 REFERENCES

ASTM (1997). Standard practice E 679-91. Determination of odor and taste thresholds by an ascending concentration series method of limits. Philadelphia, USA: American Society for Testing and Materials.

Brown, R. C., Sefton, M. A., Taylor, M. A., Taylor, D. K. & Elsey, G. M (2006) An odour detection threshold determination of all four possible stereoisomers of oak lactone in a white and red wine. *Australian Journal of Grape and Wine Research*. **12**, 115 - 118.

Chatonnet, P., Dubourdieu, D. & Boidron, J. (1995) The influence of *Brettanomyces*/*Dekkera* sp. yeasts and lactic acid bacteria on the ethylphenol content of red wines. *American Journal of Enology and Viticulture.* **46**, 463 - 468.

Chatonnet, P., Dubourdieu, D., Boidron, J. & Lavigne, V. (1993) Synthesis of volatile phenols by *Saccharomyces cerivisae* in wines. *Journal of the Science of Food and Agriculture*. **62**, 191 - 202.

Chatonnet, P., Dubourdieu, D., Boidron, J. & Pons, M. (1992) The origin of ethylphenols in wines. *Journal of the Science of Food and Agriculture*. **60**, 165 - 175.

Culleré, L., Escudero, A., Cacho, J., Ferreira, V. (2004) Gas chromatography-ilfactometry and chemical quantitative study of the aroma of six premium quality Spanish aged red wines. *Journal of Agricultural and Food Chemistry*. **52**, 1653 - 1660.

Curtin, C. Bramley, B. Cowey, G. Holdstock, M. Kennedy, E. Lattey, K. Coulter, A. Henschke, P. Francis, L. Godden, P. Sensory perceptions of 'Brett' and relationship to consumer preference. Blair, R.J.; Williams, P.J.; Pretorius, I.S. Proceedings of the thirteenth Australian wine industry technical conference, 29 July-2 August 2007, Adelaide, SA. : 207-211; 2008

Curtin, C. D., Bellon, J. R., Coulter, A. D., Cowey, G. D., Robinson, E. M. C., de Barros Lopes, M. A., Godden, P. W., Henschke, P. A. & Pretorius, I. S. (2005) The six tribes of "Brett" in Australia - Distribution of genetically divergent *Dekkera bruxellensis* strains across

Australian winemaking regions. *Australian and new Zealand Wine industry Journal*. **20**, 28 - 35.

Dacremont, C. & Sauvageot, F. (1997) Are replicate evaluations of triangle tests during a session good practice? *Food Quality and Preference*. **8**, 367 - 372.

du Toit, W. J., Pretorius, I. S. & Lonvaud-Funel, A. (2005) The effect of sulphur dioxide and oxygen on the viability and culturabilty of a strain of Acetobacter pasteurianus and a strain of *Brettanomyces bruxellensis* isolated from wine. *Journal of Applied Microbiology*. **98**, 862 – 871.

Escudero, A., Campo, E., Fariña, L., Cacho, J. & Ferreira, V. (2007) Analytical characterization of the aroma of five premium red wines. Insights into the role of odor families as the concept of fruitiness of wines. *Journal of Agricultural and Food Chemistry*. **55**, 4501 - 4510.

Fariña, L., Boido, E., Carrau, F. & Dellacassa, E. (2007) Determination of volatile phenols in red wines by dispersive liquid-liquid microextraction and gas chromatography-mass spectrometry detection. *Journal of Chromatography A*. **1157,** 46 - 50.

Ferreira, V., López, R. & Cacho, J. F. (2000) Quantitative determination of the odorants of young red wines from different grape varieties. *Journal of the Science of Food and Agriculture*. **80,** 1659 - 1667.

Francis, I. L. & Newton, J. L (2005) Determining wine aroma from compositional data. In: *AWRI – Advances in wine science* (edited by R. J. Blair, M. E. Francis & I. S. Pretorius A. L.), Pp 201 - 212. Glen Osmond, Australia: Australian Wine Research Institute.

Fugelsang, K. C. & Zoecklein, B. W. (2003) Population dynamics and effects of *Brettanomyces bruxellensis* strains on Pinot noir (Vitis vinifera L.) wines. *American Journal of Enology and Viticulture*. **54**, 294 - 300.

Grosh, W. (2001) Evaluation of the key odorants of foods by dilution experiments, aroma models and omission. *Chemical Senses*. **26**, 533 - 545.

Guadagni, D. G., Buttery, R. G., Okano, S. & Burr, H. K. (1963) Additive effect of sub-threshold concentrations of some organic compounds associated with food aromas. *Nature*. **200**, 1288-1289.

Guth, H (1997) Quantification and sensory studies of character impact odorants of different white wine varieties. *Journal of Agricultural and Food Chemistry*. **45**, 3027-3032.

Henschke, P., Bellon, J., Capone, D., Coulter, A., Cowey, G., Cozzolino, D., Curtin, C., Field, J., Gishen, M., Graves, P., Latey, K., Robinson, E., Francis, I. L., Lopes, M, D. & Godden, P. (2004) Incidence and control of *Brettanomyces*, the Australian perspective. *American Journal of Enology and Viticulture*. **55**, 304A.

Hesford, F. & Schneider, K. (2004) Discovery of a third ethylphenol contributing to *Brettanomyces* taint. *Obst- und Weinbau*. **140**, 11-13.

Hesford, F., Schneider, K., Porret, N. & Gafner, J. (2004) Identification and analysis of 4-ethyl catechol in wines tainted by *Brettanomyces* off-flavor. *American Journal of Enology and Viticulture*. **55**, 304A.

Laing, D. G. (1988) Relationship between the differential adsorption of odorants by the olfactory mucus and their perception in mixtures. *Chemical Senses*. **13**, 463 - 471.

Larcher, R., Nicolini, G., Bertoldi, D. & Nardin, T. (2008) Determinination of 4-ethylcatechol in wine by high-performance liquid chromatography-coulometric electrochemical array detection. *Analytica Chimica Acta*. **609**, 235 - 240.

Laska, M. & Hudson, R. (1991) A comparison of the detection thresholds of odour mixtures and their components. *Chemical Senses*. **16**, 651 - 662.

Lawless, H. T. & Heymann, H. (1998). *Sensory evaluation food, principles and practice*. New York, USA: Chapman and Hall.

Lawless, H. T. (1999) Descriptive analysis of complex odors: reality, model or illusion. *Food Quality and Preference*. **10**, 325 - 332.

Le Berre, E., Atanasova, B., Langloi, Etiéviant, P. & Thamas-Danguin, T. (2007) Impact of ethanol on the perception of wine odorant mixtures. *Food Quality and Preference*. **18**, 901 - 908.

Licker, J. L, Acree, T. E. & Henick-Kling, T. (1999) What is "Brett" (*Brettanomyces*) flavour? A preliminary investigation. In: *Chemistry of Wine Flavour. ACS Symposium Series* (edited by A. L. Waterhouse & S. E. Ebeler), Pp 96 - 115. Washington DC: American Chemical Society.

Loureiro, V. & Malfeito-Ferreira, M. (2003) Spoilage yeasts in the wine industry. *International Journal of Food Microbiology*. **86**, 25 - 20.

Martineau, B., Acree, T. E. & Henick-Kling, T. (1995) Effect of wine type on detection threshold for diacetyl. *Food Research International*. **28**, 139 - 143.

Neyman, J. (1937) Outline of a theory of statistical estimation based on the classical theory of probability. *Philosophical Transactions of the Royal Society of London. Series A, Mathematical and Physical Sciences*. **236**, 333 - 380.

O'Mahony, M. (1995) Who told you the triangle test was simple? *Food Quality and Preference*. **6**, 227 - 738.

Rayne, S., & Eggers, N. (2007) Volatile phenols from *Brettanomyces* in barreled red wines: are they stable? And where do they reside? *The Australian & New Zealand Grapegrower & Winemaker*. **522**, 64 - 69.

Roessler, E. B., Pangborn, R. M., Sidel, J. L. & Stone, H. (1978) Expanded statistical tables for estimating significance in paired-preference, paired-difference, duo-trio and triangle tests. *Journal of Food Science*. **43**, 940 - 947.

Romano, A., Perello, M. C., Lonvaud-Funel, A., Silcard, G. & de Revel, G. (2009) Sensory and analytical re-evaluation of “Brett character”. *Food Chemistry*. **114**, 15 - 19.

Snedecor, G. W. & Cochran, W.G. (1967) Shortcut and non-parametric methods. In: *Statistical Methods,* Pp 121 – 134. Ames, Iowa, USA: The Iowa State University Press.

Wirz, D. O., Heymann, H. & Bisson, L. F. (2004) Descriptive analysis of *Brettanomyces*-infected Cabernet Sauvignon wines. *American Journal of Enology and Viticulture*. **55**, 303A.

Wood, C., Siebert, T. E., Parker, M., Capone, D. L., Elsey, G. M., Pollnitz, A. P., Eggers, M., Meier, M., Vössing, T., Widder, S., Krammer, G., Sefton, M. A. & Herderich, M. J. (2008) From wine to pepper: Rotundone, an obscure sesquiterpene, is a potent spicy aroma compound. *Journal of Agricultural and Food Chemistry*. **56**, 3738 - 3744.

Chapter 4: Sensory profiling of four separate Brett-related compounds in Pinotage red wine: 4-ethylphenol, 4-ethylguaiacol, 4-ethylcatechol and isovaleric acid

1 INTRODUCTION

Brettanomyces yeast causes the wine defect commonly known as Brett character or phenolic off-flavour, which is associated with an aroma that can be described as horsey, leathery, medicinal, smoky or savoury (Wirz *et al*., 2004; Norris, 2004). Although the microbiological characteristics of this yeast have been exhaustively studied, the current understanding of its sensory effects in wine is rather limited. Two compounds, namely 4-ethylphenol and 4-ethylguaiacol are generally accepted to be mainly responsible for the sensory effects associated with *Brettanomyces*, and their presence in wine is used as diagnostic criterion for this type of spoilage. 4-ethylphenol is associated with leather-like and Elastoplast™ descriptors, whereas 4-ethylguaiacol has been linked to medicinal, spicy and clove like descriptors (Chatonnet *et al.*, 1992).

Isovaleric acid was pointed out by Licker *et al.* (1999) as one of the most odour-active substances with regard to Brett character. However, some authors have found contrasting results, as no significant difference in isovaleric acid level could be found between wines inoculated with *Brettanomyces* and a control (Fugelsang & Zoecklein, 2003). More recently, Romano *et al.* (2009) found a significant correlation between isovaleric acid levels and sensory Brett character, and speculated that the rancid/pungent aroma of this compound may contribute to what is described as Brett character.

The sensory character of 4-ethylcatechol has been described as “horsey” (Hesford & Schneider, 2004) and smoky (Larcher *et al.*, 2008). It has, however, been found that 4-ethylcatechol does not have as an intense or recognisable sensory effect as the other volatile phenols, and it has been speculated that its sensory effect is mainly due to synergism with the other Brett-related compounds (Larcher *et al.*, 2008).

Although several studies to date have investigated the sensory effects of 4-ethylphenol and 4-ethylguaiacol (Eteiévant *et al.*, 1989; Chatonnet *et al.*, 1992; Cliff & King, 2009), no formal sensory investigation has been undertaken that includes both these compounds as well as 4-ethylcatechol and isovaleric acid. Obtaining a better understanding of their sensory effects when present individually is the preliminary step to obtaining a better understanding of their sensory interactions.

The aim of this study is therefore to investigate the separate sensory effects if 4-ethylphenol, 4-ethylguaiacol, 4-ethylcatechol and isovaleric acid when present in Pinotage red wine at varying levels. These four compounds were selected or further investigation as they were the compounds most commonly linked to *Brettanomyces* spoilage in literature. This study served as an exploratory step for the profiling of these compounds in combination, which will be presented in Chapter 5.

2 MATERIALS AND METHODS

2.1 Wine samples

Two hundred litres of Pinotage red wine was obtained from a local producer (Distell Group Ltd, Stellenbosch, South Africa) during the course of 2009 and was bottled manually at the Department of Viticulture and Oenology, Stellenbosch University, South Africa. The wine was made using standard red wine making practices and completely underwent both alcoholic and malolactic fermentation. The wine had an alcohol concentration of 11.6 % and a pH of 3.65.

After bottling, samples were taken to determine the levels of 4-ethylphenol, 4-ethylguaiacol, 4-ethylcatechol and isovaleric acid in the wine. The concentrations of 4-ethylphenol, 4-ethylguaiacol and isovaleric acid were determined using gas chromatography mass spectrometry (GC-MS) and the concentration of 4-ethylcatechol was determined using HPLC-MS/MS. All these analyses were conducted by an accredited wine analysis laboratory (Quantum Laboratories, South Africa) and the methods are described in detail in Chapter 6.

During the chemical analyses of the wine used during this study, it was found that the wine contained less than 10 µg/L of 4-ethylphenol, less than 10 µg/L of 4-ethylguaiacol, less than 10 µg/L 4-ethylcatechol, and 355 µg/L of isovaleric acid. From this data, the wine is considered to be free of ethylphenols. The level of isovaleric acid is close to the minimum of 300 µg/L found in red wines, as quoted by Francis & Newton (2005). The wine is therefore considered free from microbiological spoilage.

2.2 Chemicals and spiking

Solutions of 10 mg/mL, 1 mg/mL, 100 µg/mL and 10 µg/mL of 4-ethylphenol, 4-ethylguaiacol, 4-ethylcatechol and isovaleric acid (Aldrich, South Africa) were prepared in 99.5% ethanol (Merck Chemicals, South Africa). These solutions were used to produce wine samples spiked with the desired concentrations of each compound. This study focussed on the separate sensory effects of the four compounds, and therefore wine samples were spiked with only one of the four compounds.

The concentrations of the four compounds used were identical to those used in the central composite design (see Chapter 5). In Chapter 5, the design and the method for concentration selection is described in more detail. The choice to correspond the levels used in the singular sensory profiling (Chapter 4) with those of the central composite design (Chapter 5) was made so that the two datasets could be compared.

The concentrations used were predetermined as follows: Firstly, detection thresholds were determined as described in Chapter 3, and it was decided that level 2 in the central

composite design should correspond to the determined detection threshold. This was done to allow for the investigation of sensory effects of the compounds at, below, and above their detection thresholds. The highest level (level 5) corresponded to the highest level that is likely to occur naturally in wine. This level was decided on with the guidance of literature and following consultation with wine-industry experts (Thales, South Africa). Levels 2 and 5 were used to calculate the rest of the levels of the central composite design. Due to the inherent differences between the base wines used in Chapter 3 and this chapter, all levels used were subjected to extensive sensory pre-screening in order to determine whether they adhere to the sensory criteria set.

The central composite design levels were chosen along the same logarithmic scale as the eight levels used for the detection thresholds. The levels used during detection thresholds all fall on the curve $c = ab^{n-1}$, where c, is the concentration, a is the starting point (level 1), b is the multiplication factor and n is the level number. The levels chosen in the design were on a simple numerical scale that corresponded to that used in the detection thresholds in they also satisfy $c = ab^{n-1}$. This is shown in Figure 4.1. This was done to compensate for the fact that the distances between levels in the central composite design are predetermined. This created a challenge as the highest levels that this research wished to investigate were between five and twenty times the magnitude of the detection threshold.

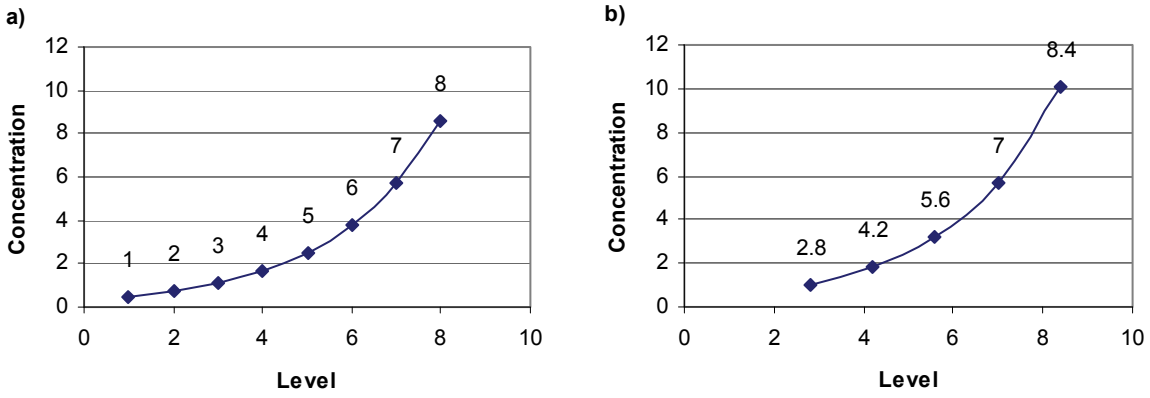


Figure 4.1. Graphical explanation of the method of level selection. Both these curves have the equation $c=ab^{n-1}$, where c is the concentration, b is the multiplication factor used, and n is the level. a) Shows the levels used during the determination of detection thresholds (Chapter 3). b) Shows the levels used in this chapter, falling along the same curve as those in a).

The use of a logarithmic scale compensated for the differences in magnitude, and allowed for the concentrations in question to be fitted to the central composite design. For this reason, two sets of levels are used in this study. The “design” levels are those determined by the design, and fall between 0 and 13 (Table 4.1). The actual concentrations used were determined by logarithmically transforming these levels and are shown in Table 4.2. In both

these tables, levels are numbered 1 to 5, and these values are used throughout this chapter when referring to samples, with reference to the concentrations and design levels where necessary.

Concentration ranges were finalised with the help of wine-industry experts by means of several sessions of consensus sensory analysis. Before commencement of the formal sensory tests, the final samples were also subjected to sensory pre-assessment, using the mentioned wine-industry experts.

Table 4.1. Design levels for spiking of wines with Brett-related compounds.

Compound	Level				
	1	2	3	4	5
4-ethylphenol	0.5	3.5	6.5	9.5	12.5
4-ethylguaiacol	0.75	2.5	4.5	6	7.75
4-ethylcatechol	2.8	4.2	5.6	7	8.4
Isovaleric acid	2.5	4.5	6.5	8.5	10.5

Table 4.2. Actual concentrations of Brett-related compounds tested.

Compound	Level				
	1	2	3	4	5
4-ethylphenol (µg/L)	82	227	623	1711	4695
4-ethylguaiacol (µg/L)	65	117	230	381	688
4-ethylcatechol (µg/L)	181	290	465	745	1193
Isovaleric acid(µg/L)	381	431	577	997	2210

2.3 Singular profiling of samples

The compounds were profiled using quantitative descriptive analysis according to the general descriptive method (Lawless & Heymann, 1998). The concentrations of the compounds are listed in Table 4.2. The samples that were analysed contained the base wine and only one of the compounds, therefore the samples were analysed in sets of six containing 5 spiked samples plus one control sample (not being spiked). The panel consisted of 10 judges who had previous experience in the use of quantitative descriptive analysis. Most of the panellists also took part in the determination of detection thresholds for these compounds (Chapter 3) and therefore already had a degree of familiarity with the compounds under investigation.

The first phase of training consisted of five sessions of general training. During the first session, the judges received all the samples, as well as a range of reference standards adapted from Noble *et al.* (1987). The purpose of this session was to familiarise the judges with the samples and descriptors used during this part of the study. During the subsequent four training

sessions, the samples were evaluated per compound, and descriptors were generated for use during quantitative descriptive analysis.

The second phase of training consisted of two session of training per compound (therefore eight sessions in total). During the first training session, descriptors were finalised, and an attempt was made to finalise the positions of the samples on a 100 mm-unstructured line scale. During the second training session, the positions of the different samples on the unstructured line scale were finalised, and an attempt was made to reach consensus regarding the different samples.

The descriptors used in the final descriptive analysis, as well as the reference standards used to define them, are shown in Table 4.3.

Table 4.3. Reference standards and descriptors used during singular profiling of wines spiked with variable concentrations 4-ethylphenol, 4-ethylguaiacol, 4-ethylcatechol and isovaleric acid.

Descriptor	Definition	Reference standard	Compound relevant for descriptor
Berry-like	Typical wine-sweet berry-like aroma	Control samples	All
Sick-sweet	Atypical wine-sweet – sweet smell that is uncharacteristic/unnatural for wine	None	All
Elastoplast™[a]	Smell associated with Band-Aid™or Elastoplast™	1 piece of Elastoplast™ bandage	4-ethylphenol
Leather	The smell associated with leather	1 piece of leather	4-ethylphenol
Smoky	Smoky smell associated with wine	Wine spiked with “smoke essence” used in meat products (3 drops in 100 mL wine)	4-ethylguaiacol
Medicinal/ Listerine-like	Minty smell associated with mouthwash	Wine spiked with Listerine™, a mouthwash (3 drops in 100 mL wine)	4-ethylguaiacol
Savoury	Meaty smell associated with food	Wine spike with soy sauce (1 mL in 100 mL wine)	4-ethylcatechol
Pungent	Sweaty/ rancid/ cheesy/ vinegary	A small piece of mild blue cheese (Simonzola™)	Isovaleric acid

[a]Throughout this study, the term “Elastoplast™” is used. This term is the local equivalent of “Band-Aid™”.

Each compound was analysed during four sessions of descriptive analysis (16 session in total). The final profiling analyses were conducted by 10 trained assessors in booths with standard artificial daylight lighting and temperature control at 20°C ±1°C. The wine was analysed in standard ISO wine tasting glasses, sample size was 20 mL and samples were served at 20°C ±1°C. Prior to the analysis, the wine glasses containing the wine samples were

covered with plastic lids. This prevented the aroma of the wine from escaping or contaminating the laboratory environment. Samples were marked with a random three-digit code, and were randomised within the judges. However, the non-spiked control sample was labelled “C”, and was always presented in the first position.

2.4 Data analysis

A randomized complete block design was used for the sensory analysis where each judge received a control sample containing only the base wine and five spiked samples. This was replicated four times. The data were analysed using SAS® software (Version 9; SAS Institute Inc, Cary, USA) and subjected to the Shapiro-Wilk test for non-normality of the residuals (Shapiro & Wilk, 1965). If non-normality was found to be significant ($P \leq 0.05$) and caused by skewness, the outliers were identified and removed until the data were normal or symmetrically distributed (Glass *et al.,* 1972). Using line plots indicating temporal stability and internal consistency, single odd judges were identified and removed. The final analysis of variance (ANOVA) was performed after the above-mentioned procedures. Student’s t-least significant difference (LSD) was calculated at the 5% significance level to compare treatment means. Discriminant analysis and Principal Component Analysis (PCA) were performed on responses for the different judges of the different levels. Multivariate data analyses were performed using the XLStat software (Version 2009.5.0.1, Addinsoft, SARL, Paris, France).

3 RESULTS AND DISCUSSION

3.1 4-ethylphenol

The overall trends found during the profiling of 4-ethylphenol are shown in Figure 4.2, and the mean values with least significant differences are shown in Table 4.4. As can be seen in this figure and table, there was a large decrease in berry-like character when 4-ethylphenol is added at levels below its detection threshold (level 2). There was also a significant increase in the levels of the Elastoplast™ and sick-sweet descriptors. However, there were no significant changes between the first addition, detection threshold and the level above detection threshold, in terms of the sick-sweet and Elastoplast™ descriptors. The berry-like character continued to decrease up to level 4 (1711 µg/L). The sick-sweet and Elastoplast™ descriptors increased significantly from level 3 (623 µg/L) to level 4 (1711 µg/L), and from level 4 (1711 µg/L) to level 5 (4695 µg/L). In terms of the leather descriptor, there was no real difference between the non-spiked control samples and additions of level 1 (82 µg/L), 2 (227 µg/L), or 3 (62 3µg/L).

Although there was a significant difference ($p \leq 0.05$) between the non-spiked sample and level 2, there was no difference between this sample and level 1, and level 3 and level 1. However, the level of leather character increases significantly from an addition of level 3 to level 4, and from level 4 to level 5.

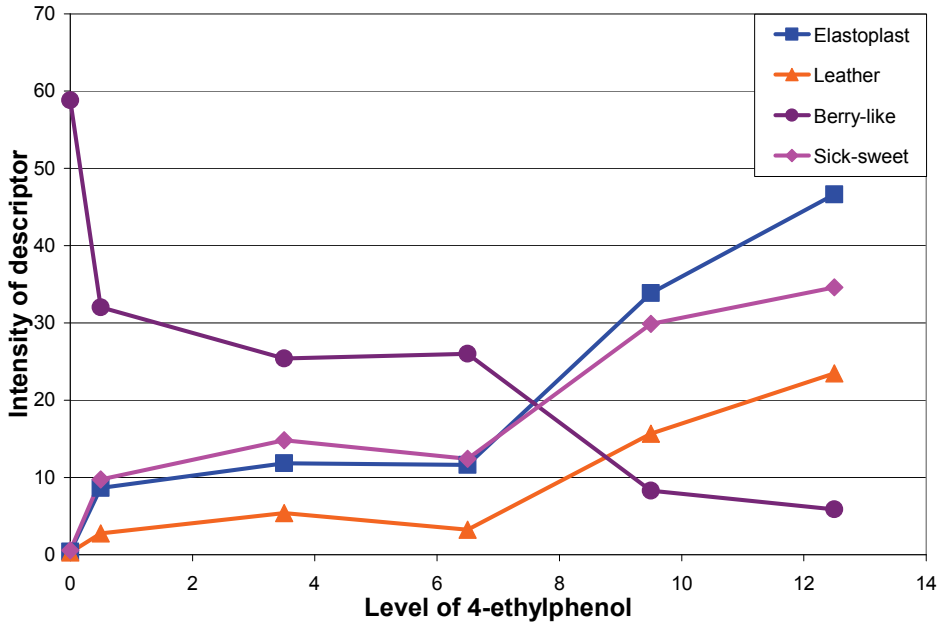


Figure 4.2. Change in sensory profile of Pinotage wine due to the addition of 4-ethylphenol. Levels are "design levels".

Table 4.4. Change in sensory profile of Pinotage wine due to the addition of 4-ethylphenol.

Level (conc)	Berry-like[1]	Sick-sweet[1]	Elastoplast™[1]	Leather[1]
0(0 µg/L)	58.8 ^{a}	0.6 ^{e}	0.4 ^{d}	0.3 ^{d}
1(82 µg/L)	32.0 ^{b}	9.8 ^{d}	8.6 ^{c}	2.8 $^{c\,d}$
2(227 µg/L)	25.4 ^{c}	14.8 ^{c}	11.8 ^{c}	5.4 ^{c}
3(623 µg/L)	26.0 ^{c}	12.4 $^{c\,d}$	11.6 ^{c}	3.2 ^{d}
4(1711 µg/L)	8.3 ^{d}	29.9 ^{b}	33.8 ^{b}	15.7 ^{b}
5(4695 µg/L)	5.9 ^{d}	34.6 ^{a}	46.6 ^{a}	23.4 ^{a}
Least Significant Difference* (p = 0.05)	**4.56**	**3.65**	**4.70**	**3.20**

[1] Values with the same superscript are not significantly different (p = 0.05).

From this it can be assumed that the effect of 4-ethylphenol manifests itself as a suppression of natural berry-like character below level 4, which results in a sick-sweet character. The typical Elastoplast™ and leather-like aromas associated with this compound are only significantly present from concentrations higher than level 4 (1711 µg/L).

Figure 4.3 shows the results of the discriminant analysis performed on the different levels of 4-ethylphenol. The first two factors explained 99% of the variance. Figure 4.3 shows a clear separation between levels 4 and 5 and level 0, the non-spiked sample. There is also a

separation between level 0 and level 3. From this it can be seen that judges could detect a difference with an addition of level 3 of 4-ethylphenol, but that this difference only became clear from level 4. This substantiates the results found during the univariate analysis of the data.

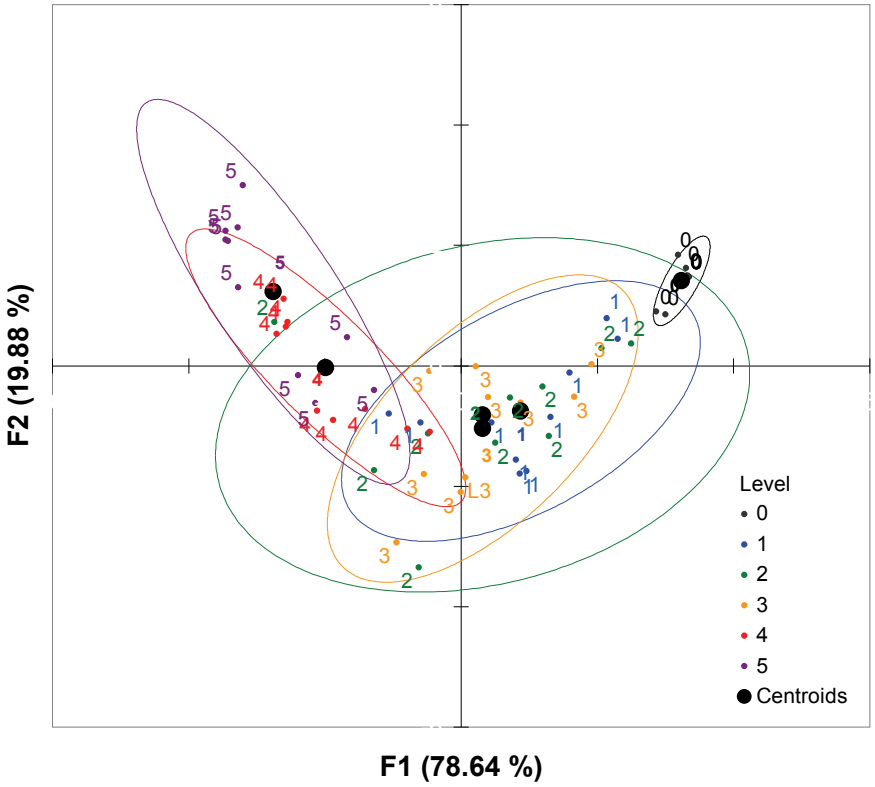


Figure 4.3. Discriminant analysis of data obtained during sensory analysis of wines spiked with 4-ethylphenol.

Figure 4.4 shows a PCA biplot of the results obtained during profiling of the samples spiked with 4-ethylphenol. 96% of the variation was described by the first two factors, with 87 and 9% of the variation being described by factors F1, and F2 respectively. This means that the samples have a more or less linear distribution along F2. It can be seen that F1 is driven by the different descriptors, with berry-like negatively associate with F1, and Elastoplast™, sick-sweet and leather positively associated with F1. Along F2, the loadings for Elastoplast™ and leather are grouped together, but are separated from sick-sweet. This can be ascribed to the slightly different pattern that the sick-sweet descriptor followed in Figure 4.2. This aroma increases more drastically with the addition of lower levels of 4-ethylphenol than the other descriptors, but does not show as large an increase above level 4. The loading for sick-sweet also shows a strong negative association to the berry-like descriptor along F1, which indicates that it arises as a result of the suppression of the natural berry-like character of the wine.

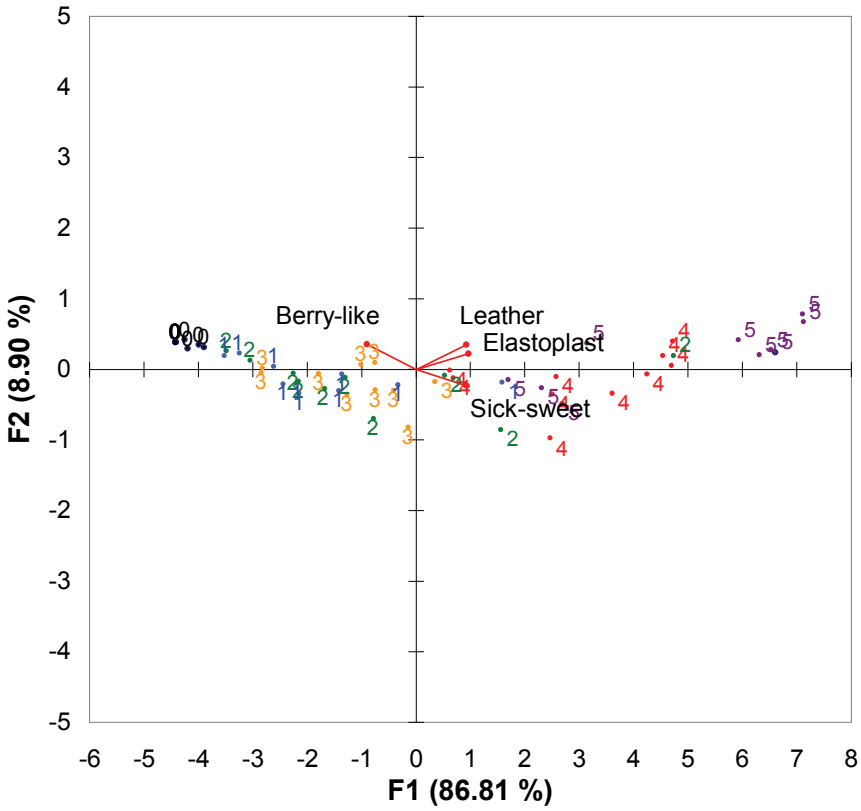


Figure 4.4. PCA biplot of all data obtained during sensory of samples containing different levels of 4-ethylphenol. Level of 4-ethylphenol is indicated by corresponding numbers.

The scores for the different levels of 4-ethylphenol also separate along F1 – levels 0 to 3 of 4-ethylphenol fall to the negative (left) side of F1, and levels 4 and 5 tend to fall to the positive (right) side of F1. From this it can be concluded that the leather, Elastoplast™ and sick-sweet characteristics are more prominent in levels 4 and 5, as these scores associate with the loadings associated with these attributes. This yet again substantiates the results found during the univariate statistical analyses, as the levels of 4-ethylphenol (levels 4 and 5) which exhibited high levels of the leather, Elastoplast™ and sick-sweet descriptors associate with the loadings for those descriptors (Table 4.4). The levels which did not show significant differences from one another in all the attributes (levels 1, 2 and 3) also associate with one another in Figure 4.4. Significant differences were however found between these samples and levels 4 and 5. This explains the separation of these samples along F1 in Figure 4.4.

The overall increase in the Elastoplast™ and leather descriptors with an increase in 4-ethylphenol concentration is expected, as these descriptors have been linked to this compound by several other authors (Chatonnet *et al*., 1992; Curtin *et al*., 2008). However, the “stable” and “barnyard” descriptors that have also been linked to this descriptor could not be perceived or quantified by the panel. This may be due to two reasons. Firstly, it is likely that these aromas could not be recognised by the panel due to a lack of internal reference for these aromas caused by a lack of exposure to these odours (Hughson & Boakes, 2002). It is also possible that the “stable” and “barnyard” descriptors are a result of interaction of different compounds

associated with this defect. Although such blending phenomena have not yet been explored in wine, they have been found by Brodin *et al*. (2007) and Le Berre *et al*. (2008) in other odourant mixtures.

3.2 4-ethylguaiacol

The overall results obtained during the profiling of Pinotage spiked with 4-ethylguaiacol are shown in Figure 4.5. The mean values of the samples and their least significant differences are shown in Table 4.5.

Table 4.5. Change in sensory profile of Pinotage wine due to the addition of 4-ethylguaiacol.

Level(conc)	Berry-like[1]	Sick-sweet[1]	Medicinal[1]	Smoky[1]
0(0 µg/L)	59.4 ^{a}	1.9 ^{e}	1.6 ^{d}	3.3 ^{e}
1(65 µg/L)	35.2 ^{b}	12.9 $^{b\,c}$	5.5 ^{c}	12.6 $^{c\,d}$
2(117 µg/L)	33.9 ^{b}	9.1 ^{d}	6.2 ^{c}	10.1 ^{d}
3(230 µg/L)	31.8 ^{b}	10.4 $^{c\,d}$	6.4 ^{c}	17.0 ^{c}
4(381 µg/L)	17.5 ^{c}	15.7 ^{b}	11.8 ^{b}	28.7 ^{b}
5(688 µg/L)	9.9 ^{d}	20.8 ^{a}	17.2 ^{a}	44.3 ^{a}
Least Significant Difference (p=0.05)	**5.24**	**3.36**	**2.30**	**5.75**

[1] Values with the same superscript are not significantly different (p = 0.05).

As is evident in Figure 4.5 and Table 4.5, a significant decrease in berry-like character occurred with an addition of 4-ethylguaiacol below its detection threshold. However, the next significant change in berry-like character only occurs at level 4 (381 µg/L), which implies that the change in this character only occurred in Pinotage red wine when the compound's concentration was significantly above it detection threshold. Similarly, a significant increase in the medicinal and smoky attributes can be observed at an addition below detection threshold. Although but there is no significant increase from below to above detection threshold, a further significant change is observed from level 3 (230 µg/L) to level 4 (381 µg/L). The sick-sweet descriptor follows a similar pattern, although here the difference occurs from addition below detection threshold to level 5 (688 µg/L). This means that the presence of 4-ethylguaiacol induced the sick-sweet attribute of the wine, but this attribute only increased in intensity when this compound was present at very high levels.

Discriminant analysis performed on the different levels of 4-ethylguaiacol shows a clear separation between level 0 and levels 3, 4 and 5 (Figure 4.6). This makes sense as there were no real significant differences in the different descriptors between the other levels, which indicates that the judges could not clearly perceive differences between the samples. For this reason, the samples are not expected to separate clearly on Figure 4.6, as the differences are too small to cause clear separation.

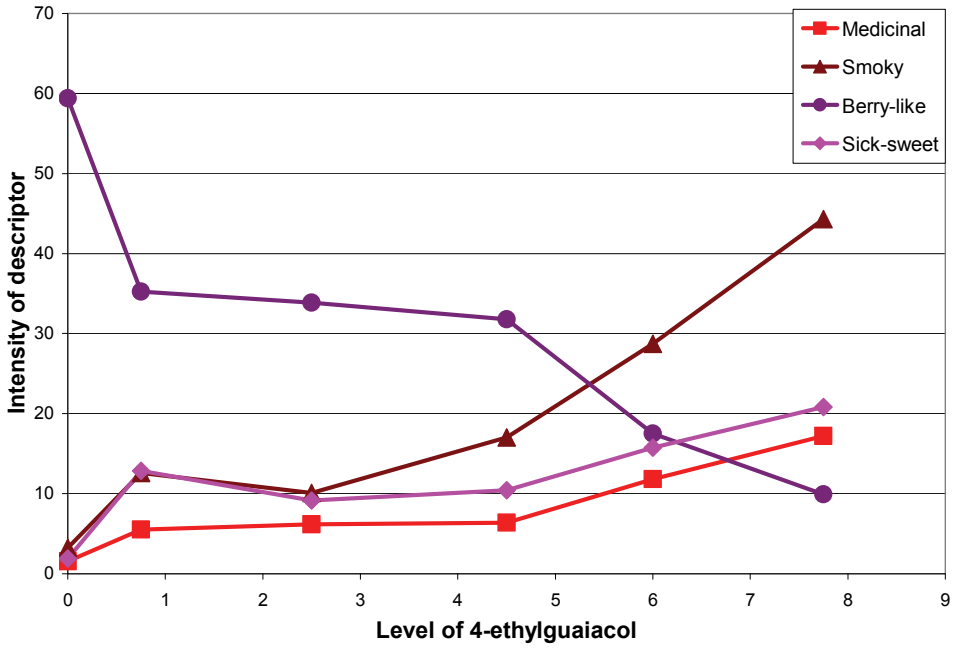


Figure 4.5. Change in sensory profile of Pinotage wine due to an induced increase in 4-ethylguaiacol level. Levels are "design levels".

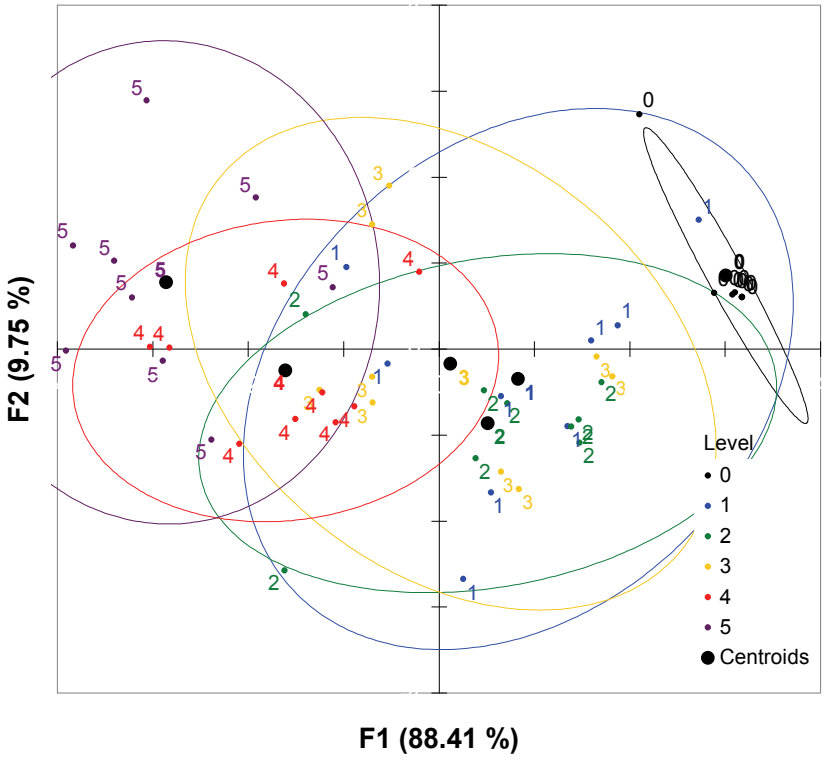


Figure 4.6. Discriminant analysis of data obtained during singular profiling of wines spiked with 4-ethylguaiacol.

Figure 4.7 shows a PCA biplot for 4-ethylguaiacol. 87% of the total variation could be explained by this biplot, 74% by F1 alone. This implies that the difference between samples is of

a near-linear nature. Similarly to what was found with 4-ethylphenol, F1 separates berry-like from the other descriptors. This is due to the fact the berry-like character decreased with an increase in concentration of 4-ethylguaiacol, whereas the other descriptors increased with an increase in concentration. F2 separates the medicinal attribute from the sick-sweet and smoky attributes. The smoky attribute associates with the sick-sweet attribute. Although this seems unexpected, both these compounds follow similar patterns in Figure 4.5, which explains their association. As in the case of 4-ethylphenol, there is a strong negative association between the loadings for sick-sweet and berry-like descriptors, which further substantiates the hypothesis that the sick-sweet characteristic arises due to a suppression of the natural berry-like character of the wine.

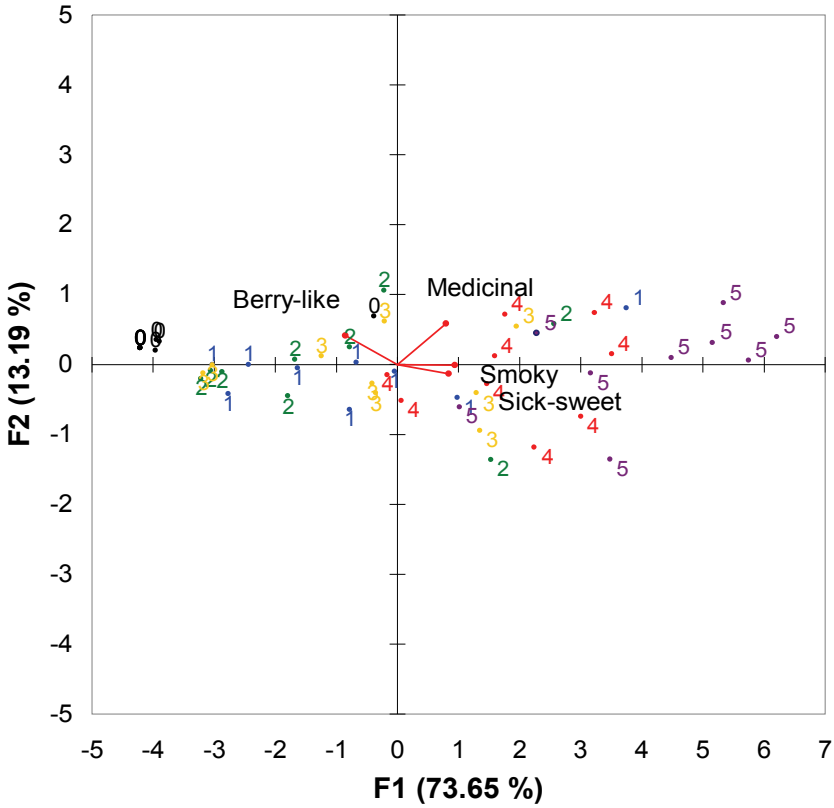


Figure 4.7. PCA biplot of all data obtained during sensory of samples containing different levels of 4-ethylguaiacol. Level of 4-ethylguaiacol is indicated by corresponding numbers PCA biplot of samples for 4-ethylguaiacol

In terms of sample scores, levels 0, 1, and 2 tend to fall to the negative side of F1, and samples 4 and 5 tend to fall towards the positive side. However, level 3 tends to fall towards the middle of the biplot, which fits in with levels 0, 1, and 2 being strongly associated with the berry-like attribute, but not with the medicinal or smoky attributes. Conversely, levels 4 and 5 were associated with the medicinal and smoky attributes, which explains their positions. The distribution of level 3 can be explained by looking at Figure 4.6, which indicates a poor discrimination between this level and all the other levels except level 0.

Overall, the increase in the medicinal aroma caused by an increase in 4-ethylguaiacol is expected, as this aroma has been linked with Brett character in literature (Curtin *et al*., 2008). However, the clove-like or spicy aroma that is commonly linked to this compound (Chattonet *et al*., 1992; Licker *et al*., 1999; Curtin *et al*., 2008) could not be accurately identified in the panel. This was in spite of the fact that the reference standard for the clove-like aroma (Noble *et al*., 1987) was employed. This may be due to the fact that the Medicinal and the clove-like descriptors were cognitively similar, which prevented the panel from distinguishing between discriminating between these aromas (Escudero *et al*., 2007). Finally, the smoky attribute coupled to this compound in this study has not been previously linked with 4-ethylguaiacol or wines spoiled with *Brettanomyces*. This is further indication of the sensory complexity of Brett character.

3.3 4-ethylcatechol

A summary of the results obtained for 4-ethylcatechol are shown in Table 4.6 and Figure 4.8.

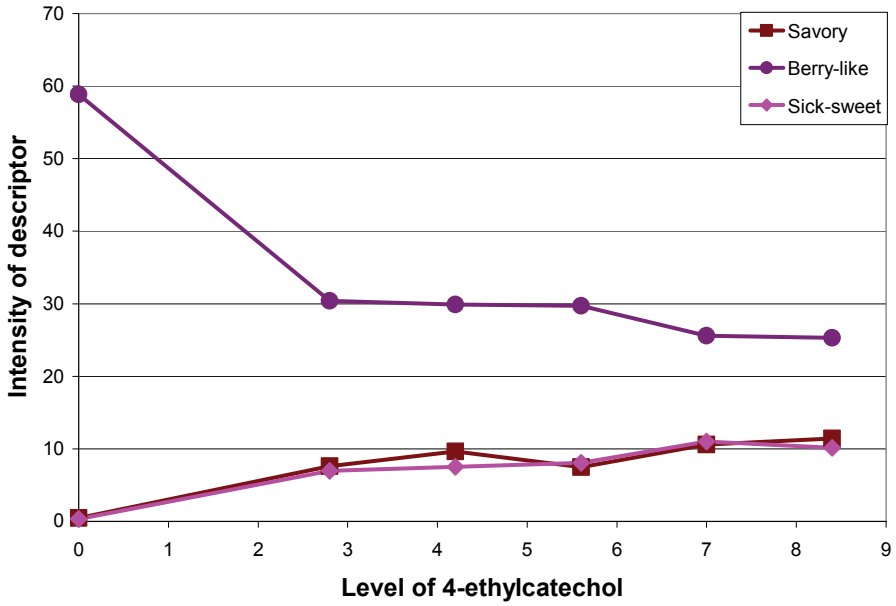


Figure 4.8. Effect of a change in 4-ethylcatechol level on the sensory profile of Pinotage wine. Levels are “design levels”.

Figure 4.8 and Table 4.6 show that the addition of 4-ethylcatechol decreases the berry-like character of a wine when it is added below detection threshold, but does not significantly affect this attribute with further additions. The sick-sweet characteristic follows a similar pattern, but there is a significant difference between an addition below detection threshold (290 µg/L) and levels 4 (745 µg/L) and 5 (1193 µg/L). In terms of savoury character, the addition below

detection threshold also shows a significant change, but levels 4 and 5 are significantly different from additions below detection threshold. The results seem to indicate that 4-ethylcatechol on its own not does significantly affect the overall profile of the wine to the same degree as 4-ethylphenol and 4-ethylguaiacol, as predicted by Larcher *et al.* (2008). Considering that the a 100 mm scale was used, the change in berry-like character is approximately 35 out of a possible 100 – which is a significant change but still significantly less than the 50 change caused by the addition of 4-ethylphenol and 4-ethylguaiacol. In addition, the maximum levels of the sick-sweet and savoury characteristics were approximately 10 out of a possible 100, which is generally termed as just detectable.

Figure 4.9 shows the results for the discriminant analysis of the data for 4-ethylcatechol. Level 0 separates from levels 4 and 5, but the rest of the levels neither separate from one another nor from Level 0. This implies that the panel experienced difficulty in judging the subtle differences between the lower levels of 4-ethylcatechol. This configuration is also mirrored by the groupings in terms of least significant differences shown in Table 4.6

A PCA biplot of the data obtained during the profiling of 4-ethylcatechol is shown in Figure 4.10. 94% of the variation is explained by the first two components, F1 and F2 account for 83 and 11% respectively. Similar to 4-ethylphenol and 4-ethylguaiacol, F1 separates the berry-like descriptor from the savoury and sick-sweet attributes. In Figure 4.10, the sick-sweet and savoury attributes are differentiated along F2. A strong negative association between the sick-sweet and berry-like attributes (like in the cases of 4-ethylphenol and 4-ethylguaiacol) could not be observed. However, when looking at Figure 4.8, it can be seen that the decrease in berry-like character is much more severe than the increase in sick-sweet character, where in the cases of 4-ethylphenol and 4-ethylguaiacol there was a similar substantial increase. This may indicate that although 4-ethylcatechol suppresses berry-like character in wine, its elusive character involves interaction with the production of sick-sweet aromas through the suppression of berry-like character. Although the scores are arranged in a linear-like fashion, the only clear pattern that can be seen is the separation of the non-spiked samples from the spiked samples. This substantiates the pattern found in the discriminant analysis and the univariate analyses.

Table 4.6. Change in sensory profile of Pinotage wine due to changes in 4-ethylcatechol level.

Level(conc)	Berry-like[1]	Sick-sweet[1]	Savoury[1]
0(0 µg/L)	58.9 ^{a}	0.3 ^{d}	0.5 ^{c}
1(181 µg/L)	30.4 ^{b}	7.0 ^{c}	7.6 ^{b}
2(290 µg/L)	29.9 ^{b}	7.5 $^{b c}$	9.6 $^{a b}$
3(465 µg/L)	29.7 ^{b}	8.1 $^{a b c}$	7.5 ^{b}
4(745 µg/L)	25.6 ^{b}	11.0 ^{a}	10.6 ^{a}
5(1193 µg/L)	25.3 ^{b}	10.1 $^{a b}$	11.4 ^{a}
Least Significant Difference (p=0.05)	**5.35**	**2.97**	**2.69**

[1] Values with the same superscript are not significantly different (p = 0.05).

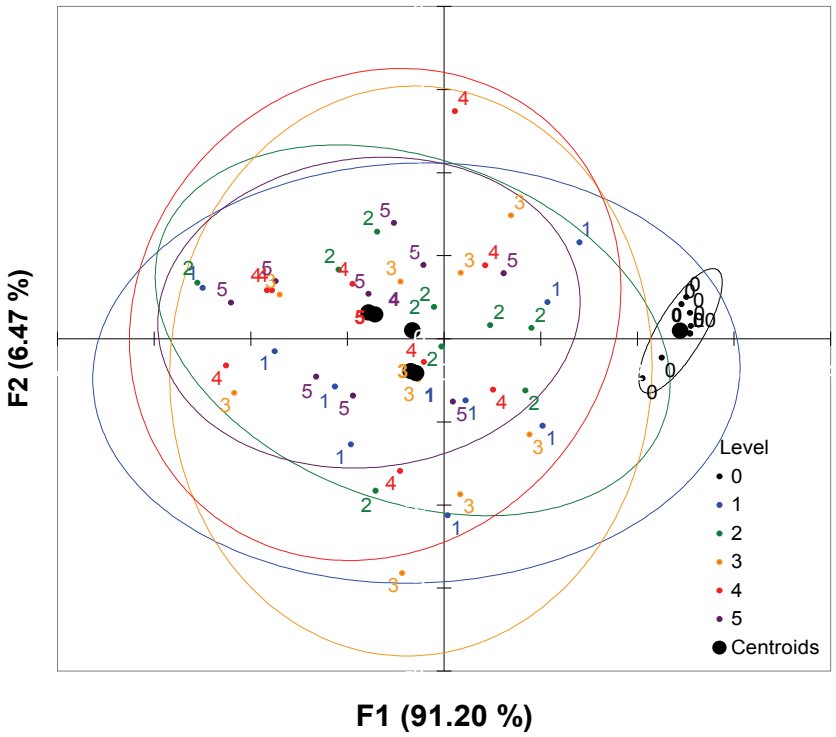


Figure 4.9. Discriminant analysis of all data obtained during sensory profiling of Pinotage spiked with different levels of 4-ethylcatechol.

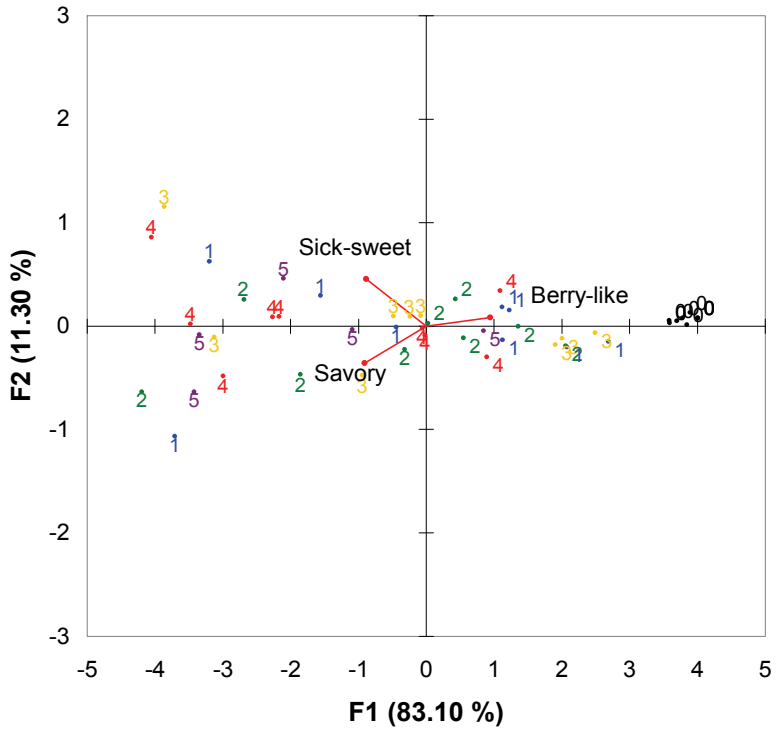


Figure 4.10. PCA biplot of all data obtained during sensory profiling of samples containing different levels of 4-ethylcatechol. Level of 4-ethylcatechol is indicated by corresponding numbers.

It is interesting to note that neither of the descriptors linked to 4-ethylcatechol in literature – namely "smoky" (Larcher *et al*., 2008; Curtin *et al*., 2008) and "horsey" (Hesford *et al*., 2004) – were found during this study. However, the savoury attribute could be similar to the "soy" descriptor that was coupled to *Brettanomyces*-infected wines by Wirz *et al.* (2004). The savoury attribute is closely related to all three these attributes. The findings of this study regarding the sensory effects of 4-ethylcatechol can therefore be regarded to be in line with literature.

3.4 Isovaleric acid

In a similar fashion to the other compounds, a significant decrease in berry-like character was observed when isovaleric acid was added to the wine at low concentrations (Figure 4.11 and Table 4.7). However, the only other significant decrease in berry-like character was seen at an addition of level 5 (2210 µg/L). The sick-sweet character also increased from an addition below detection threshold, with the next two significant changes occurring at levels 4 (997 µg/L) and 5 (2210 µg/L). The pungent aroma also increased significantly with an addition below the detection threshold, and again increased significantly with an addition of levels 4 and level 5.

Figure 4.12 shows the discriminant analysis results for the sensory profiling for isovaleric acid. Level 0 is separated from all the levels except for level 2. Although none of the other levels are separated on this figure, near separation is observed between level 3 and 5. These two classes are spanned by level 4. This implies that although levels 3 and 5 are different, level 4 shares properties with both these levels. This may have been caused by the lack of significant difference between level 3 and level 4 in terms of both the berry-like and sick-sweet characteristics.

Table 4.7. Change in sensory profile due to an increase in isovaleric acid.

Level(conc)	Berry-like[1]	Sick-sweet[1]	Pungent[1]
0(355 µg/L)	60.0 ^{a}	0.1 ^{d}	0.0 ^{d}
1(381 µg/L)	35.7 ^{b}	9.4 ^{c}	13.0 ^{c}
2(431 µg/L)	34.9 ^{b}	6.9 ^{c}	11.9 ^{c}
3(577 µg/L)	37.5 ^{b}	9.9 $^{b\,c}$	13.0 ^{c}
4(997 µg/L)	33.9 ^{b}	12.7 ^{b}	18.7 ^{b}
5(2210 µg/L)	26.2 ^{c}	15.8 ^{a}	25.6 ^{a}
Least Significant Difference (p=0.05)	**4.45**	**3.03**	**3.92**

[1] Values with the same superscript are not significantly different (p = 0.05).

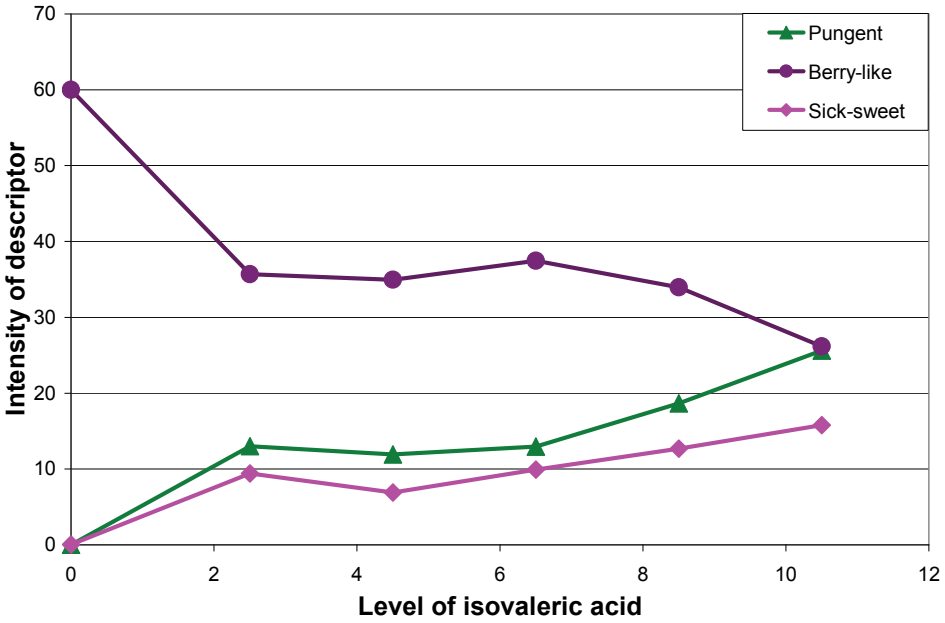


Figure 4.11. Change in sensory profile due to the increase in isovaleric acid. Levels are "design levels".

The results of PCA performed on the data obtained during the profiling of isovaleric acid can be seen in Figure 4.13. 94% of the variance could be explained by the first two components, with F1 explaining 85% of the variation and F2 explaining 9%. Similar to the other compounds, F1 is characterised by the loading for berry-like on the negative side and sick-sweet and pungent on the positive side. F2 is characterised by the separation of the sick-sweet and berry-like attributes from the pungent attribute. It is interesting to note that berry-like and sick-sweet do not have the strong negative association that was shown in the case of 4-ethylphenol and 4-ethylguaiacol. However, the pungent and berry-like attributes do show a strong negative association. This makes some sense as the lines for these two attributes in Figure 4.11 seem to be mirror-images of one another – a decrease in the berry-like attribute is associated with a comparable increase in the pungent attribute. This inversion of aroma character compared to the other compounds may be attributed to the overpowering nature of the pungent attribute, making it difficult for the judges to identify the sick-sweet characteristic in these samples.

The scores for level 1, 2 and 3 are distributed all over the biplot, and do not associate with either of the other levels or each other. This is probably because of the lack of clear difference between these three samples, which could be seen from the univariate results.

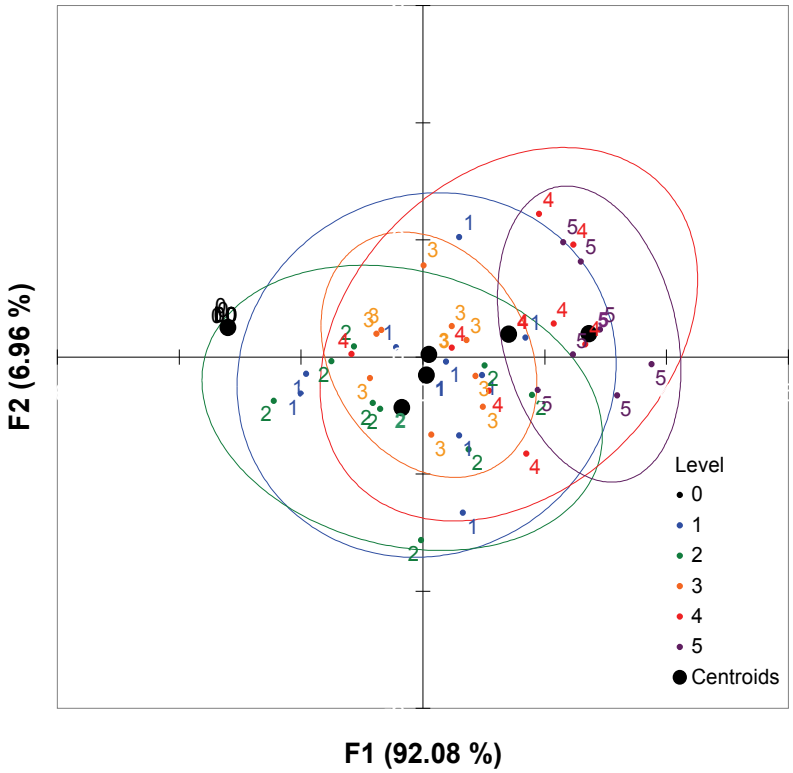


Figure 4.12. Results of discriminant analysis performed on all data obtained during the sensory profiling of Pinotage spiked with different levels of isovaleric acid.

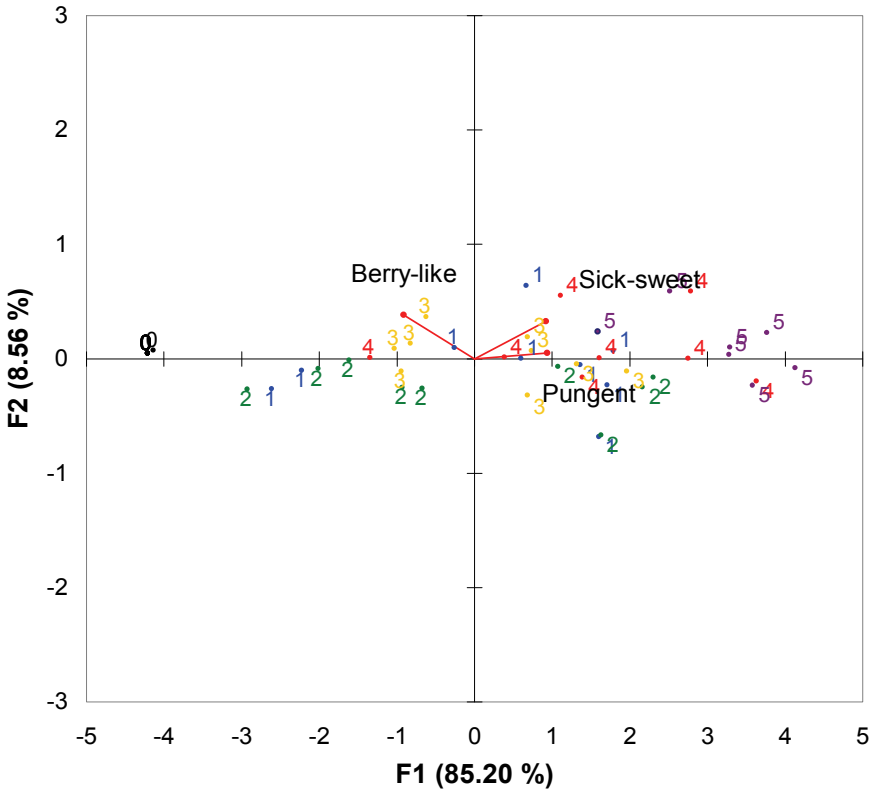


Figure 4.13. PCA biplot of all data obtained during sensory of samples containing different levels of isovaleric acid. Level of isovaleric acid is indicated by corresponding numbers.

Previous investigations into the sensory effects of isovaleric acid in wine linked this compound with “rancid” (Licker *et al*., 1999; Romano *et al*., 2008) and “sweaty” (Romano *et al*., 2009) descriptors. The pungent attribute used in this study aimed to include these two descriptors. The sensory results of this study regarding isovaleric acid are therefore in line with that which is expected from literature.

3.5 Overall discussion of common descriptors

Several general trends could be observed in terms of the sensory effect of these four Brett-related compounds. Firstly, all four compounds caused suppression in berry-like character when they were added at concentrations below their detection thresholds. Secondly, the only significant difference in berry-like character between level 1 (below detection threshold) and level 2 (detection threshold) were found for 4-ethylphenol. However, there were no significant changes in berry-like character between level 2 (approximately detection threshold) and level 3 (above detection threshold) for any of the compounds. This might be due to the fact that a different Pinotage wine was used in this study than in Chapter 3.

In some of the cases (4-ethylphenol and 4-ethylguaiacol), there was a significant decrease in berry-like character from level 3 to level 4, and in some cases (4-ethylguaiacol and isovaleric acid) a significant decrease from level 4 to level 5. In the case of 4-ethylcatechol, there was no significant change between level 2 and level 5. From these results it can be concluded that the suppressant effect on berry-like character occurs most strongly at the lower levels of 4-ethylphenol, but at the higher levels of 4-ethylguaiacol and isovaleric acid. Although its presence caused a suppression of berry-like character, 4-ethylcatechol does not seem to have a severe effect on the suppression of berry-like character with an increase in concentration.

The sick-sweet descriptor followed an opposite general trend to the berry-like descriptor for all compounds: There was an increase in sick-sweet character at additions below detection threshold, but not always a significant increase to detection threshold (level 2) or from level 2 to level 3 (detection threshold to above detection threshold). 4-ethylguaiacol did not follow this pattern strictly, as the mean for this attribute at level 2 was significantly higher than that of level 3. This can be ascribed to the fact that the medicinal (or sometimes minty) character of 4-ethylguaiacol could easily be mistaken for sick-sweet, causing interference in the analysis of this descriptor. For the other three compounds, there were significant differences between level 1 and level 4 in all the cases, with further significant differences between levels 4 and 5 for 4-ethylphenol, 4-ethylguaiacol and isovaleric acid. 4-ethylphenol caused the greatest increase in sick-sweet character, whereas 4-ethylcatechol only caused slight increases.

In terms of the other descriptors – where different descriptors are relevant for different compounds – it is interesting to note that most of the descriptors followed a similar trend to the sick-sweet descriptor (and the opposite of the berry-like descriptor): an increase from level 0 to level 1, no increase between levels 1 and 3, and an increase from level 3 to 4 and/or level 4 to 5. From these data it can be concluded that the berry-like descriptor character decreases with increase of the “taint” descriptors.

4 CONCLUSIONS

As expected from literature (Licker *et al.*, 1999; Fugelsang & Zoecklien, 2003; Ugarte *et al.*, 2005; Fariña *et al.*, 2007; Cliff & King, 2009), the four different Brett-related spoilage compounds all suppressed berry-like character in wines, although not to an equal extent. The increase in the relevant descriptors for 4-ethylphenol, 4-ethylguaiacol and isovaleric acid were expected, as these compounds had been linked with these attributes in literature (Chatonnet *et al*., 1992; Licker *et al*., 1999; Curtin *et al*., 2008; Romano *et al*., 2009). The slight increase in savoury character caused by 4-ethylcatechol is in agreement with the findings of Larcher *et al.* (2008) and Hesford *et al*. (2004).

When profiling these components by themselves, it was found that all four compounds suppressed berry-like character, and caused an increase in an atypical sick-sweet (or confected) character. In the cases of 4-ethylphenol and 4-ethylguaiacol, it can be concluded from the results of multivariate statistics (Figure 4.4 and Figure 4.7) that this attribute is a direct result of the suppression of fruitiness. Although this is also evident for 4-ethylcatechol and isovaleric acid, this conclusion cannot be drawn for these compounds, as other factors seem to influence the presence of the sick-sweet characteristic. As this attribute has not been coupled to *Brettanomyces* spoilage to date, it could be speculated that although it is present with the individual compounds, sensory interactions between these compounds negate this effect. Several other authors (Chatonnet *et al*., 1993; Fugelsang & Zoecklein, 2003; Curtin *et al*., 2008; Romano *et al*., 2009) have also speculated about the sensory interactions evident between these compounds. This requires further investigation.

In this study, PCA has shown itself to be a more powerful tool for the interpretation of sensory data than normal univariate plots and least significant differences, as it provided insight into the relationships between different samples and attributes. Although the nature of the experiments in this study were relatively simple, with one principal component explaining the majority of variance in all the cases, the use of PCA was still insightful for the overall interpretation. The exploratory use of PCA in sensory data is therefore recommended as a complementary tool for univariate techniques in future studies.

5 REFERENCES

Brodin, M., Moeller, P. & Olsson, M. (2007) Are we mixing odorants or odors? *Chemical Senses*. **32**, A19.

Chatonnet, P., Dubourdieu, D., Boidron, J. & Pons, M. (1992) The origin of ethylphenols in wines. *Journal of the Science of Food and Agriculture*. **60**, 165 - 175.

Cliff, M. A. & King, M. C. (2009) Influence of serving temperature and wine type on perception of ethyl acetate and 4-ethylphenol in Wine. *Journal of Wine Research*. **20**, 45 - 52.

Curtin, C. D., Bellon, J. R., Coulter, A. D., Cowey, G. D., Robinson, E. M. C., de Barros Lopes, M. A., Godden, P. W., Henschke, P. A. & Pretorius, I. S. (2005) The six tribes of "Brett" in Australia - Distribution of genetically divergent *Dekkera* bruxellensis strains across Australian winemaking regions. *Australian and new Zealand Wine industry Journal*. **20**, 28 - 35.

Eteiévant, P. X., Issanchou, S. N., Marie, S., Ducruet, V. & Flanzy, C. (1989) Sensory impact of volatile phenols on red wine aroma: influence of carbonic maceration time and storage. *Sciences des aliments*. **9**, 19 - 31.

Fariña, L., Boido, E., Carrau, F. & Dellacassa, E. (2007) Determination of volatile phenols in red wines by dispersive liquid-liquid microextraction and gas chromatography-mass spectrometry detection. *Journal of Chromatography A.* **1157**, 46 - 50.

Francis, I. L. & Newton, J. L (2005) Determining wine aroma from compositional data. In: *AWRI – Advances in wine science* (edited by R. J. Blair, M. E. Francis & I. S. Pretorius A. L.), Pp 201 - 212. Glen Osmond, Australia: Australian Wine Research Institute.

Fugelsang, K. C. & Zoeklein, B. W. (2003) Population dynamics and effects of *Brettanomyces bruxellensis* strains on Pinot noir (Vitis vinifera L.) wines. *American Journal of Enology and Viticulture*. **54**, 294 - 300.

Glass, G.V., Peckham, P.D. & Sanders, J.R. (1972). Consequences of failure to meet assumptions underlying the fixed effects analyses of variance and covariance. *Review of Educational Research*, **42**, 237 - 288.

Hesford, F. & Schneider, K. (2004) Discovery of a third ethylphenol contributing to *Brettanomyces* taint. *Obst- und Weinbau*. **140**, 11 - 13.

Hughson, A. L. & Boakes, R. A. (2002) The knowing nose: the role of knowledge in wine expertise. *Food Quality and Preference*. **13**, 463 - 472.

Larcher, R., Nicolini, G., Bertoldi, D. & Nardin, T. (2008) Determination of 4-ethylcatechol in wine by high-performance liquid chromatography-coulometric electrochemical array detection. *Analytica Chimica Acta*. **609**, 235 - 240.

Lawless, H. T. & Heymann, H. (1998). *Sensory evaluation food, principles and practice*. New York, USA: Chapman and Hall.

Licker, J. L, Acree, T. E. & Henick-Kling, T. (1999) What is "Brett" (*Brettanomyces*) flavour? A preliminary investigation. In: *Chemistry of Wine Flavour. ACS Symposium Series* (edited by A. L. Waterhouse & S. E. Ebeler), Pp 96 - 115. Washington DC: American Chemical Society.

Noble, A.C., Arnold, R.A., Buechsenstein, J., Leach, E. J., Schmidt, J. O. & Stern, P. M. (1987) Modification of a standardized system of wine aroma terminology. *American Journal of Enology and Viticulture*. **38**, 143 - 146.

Norris, L. (2004) Unraveling the mystery of *Brettanomyces* flavor. *American Journal of Enology and Viticulture*. **55**, 304A.

Romano, A., Perello, M. C., Lonvaud-Funel, A., Silcard, G. & de Revel, G. (2009) Sensory and analytical re-evaluation of “Brett character”. *Food Chemistry*. **114**, 15 - 19.

Shapiro, S. S. & Wilk, M. B. (1965). An analysis of variance test for normality (complete samples). *Biometrika*, **52**, 591-611.

Ugarte, P., Agosin, E., Bordeu, E. & Villalobos, J. I. (2005) Reduction of 4-ethylphenol and 4-ethylguaiacol concentration in red wines using reverse osmosis and adsorption. *American Journal of Enology and Viticulture*. **56**, 30 – 36.

Wirz, D. O., Heymann, H. & Bisson, L. F. (2004) Descriptive analysis of *Brettanomyces*-infected Cabernet Sauvignon wines. *American Journal of Enology and Viticulture*. **55**, 303A.

Chapter 5: Investigation into the sensory effects and interactions of four Brett-related compounds in Pinotage red wine

1 INTRODUCTION

Since its discovery as wine spoilage microorganism, *Brettanomyces* has been the subject of many microbiological investigations. However, although the spoilage of wine by *Brettanomyces* mainly affects its sensory properties, the organoleptic effects of *Brettanomyces* spoilage are still relatively poorly understood. 4-ethylphenol and 4-ethylguaiacol are generally accepted to be the most important *Brettanomyces* related spoilage compounds, but recently attention has been drawn to isovaleric acid and 4-ethylcatechol (Licker *et al.*, 1999; Fugelsang & Zoecklien, 2003; Hesford & Schneider, 2004; Hesford *et al.*, 2004; Larcher *et al.*, 2008; Romano *et al.*, 2009).

Isovaleric acid was pointed out by Licker *et al.* (1999) as one of the most odour-active substances with regard to Brett character. However, other authors (Fugelsang & Zoecklien, 2003) found no correlation between the presence of isovaleric acid and sensory Brett character. In a more recent study, Romano *et al.* (2009) found a significant correlation between isovaleric acid levels and sensory Brett character. This correlation led them to conclude that isovaleric acid is a marker for Brett character. They also found that the presence of isovaleric acid increased the detection thresholds of 4-ethylphenol and 4-ethylguaiacol. Both Romano *et al.* (2009) and Fugelsang and Zoecklein (2003) speculated about synergistic sensory effects involving the volatile phenols and isovaleric acid.

In 2004, a third ethylphenol, namely 4-ethylcatechol, was linked to *Brettanomyces* spoilage (Hesford & Schneider, 2004; Hesford *et al.*, 2004). This compound is produced by the same enzymatic pathway as 4-ethylphenol and 4-ethylguaiacol from caffeic acid as precursor (Hesford & Schneider, 2004). Its relatively recent linkage to *Brettanomyces* (in contrast to 4-ethylphenol and 4-ethylguaiacol) is ascribed to the fact that 4-ethylcatechol is less volatile than 4-ethylguaiacol and 4-ethylphenol, and therefore either requires a derivitisation step prior to GC-MS analysis or HPLC analysis (Hesford & Schneider, 2004; Hesford *et al.*, 2004, Larcher *et al.*, 2008). The sensory character of 4-ethylcatechol has been described as “horsey” (Hesford & Schneider, 2004), and smoky (Larcher *et al.*, 2008). It has, however, been found that 4-ethylcatechol does not have as intense a sensory effect as the other volatile phenols, and it has been speculated that its sensory effect is mainly due to synergism with the other Brett-related compounds (Larcher *et al.*, 2008; Curtin *et al.*, 2008). A study has found that when 4-ethylcatechol was present in combination with low levels of the other two ethylphenols, it caused a smoky aroma, and that it suppressed Brett character when 4-ethylphenol and 4-ethylguaiacol were at high levels (Curtin *et al.*, 2008).

Although the presence of excessive levels of ethylphenols have long been considered to be a wine spoilage defect (Eteiévant, 1981), and some authors (Eteiévant *et al.*, 1989; Chatonnet *et al.*, 1992) have attempted to correlate specific levels of ethyl phenols to consumer rejection of red wine, consumer analysis has only recently been performed on wines spoiled with *Brettanomyces* (Lattey *et al.*, 2007; Curtin *et al.*, 2008). It was found that wines with Brett

character are indeed less-liked than unspoiled wine, regardless of the lack of consumer knowledge of this defect. However, many people are of the opinion that a “little bit of Brett is nice”, as its presence adds to the complexity of wine (Oelofse *et al.*, 2009). Brett character is also specifically associated with some French wines and is ascribed as being part of “terroir”.

The central composite design is an experimental design method that is used as a screening design for interactions. It is specifically suited to situations where the number of samples that can be analysed is limited (Esbensen, 2002), which is a common challenge regarding sensory analysis. The central composite design has recently been used to investigate synergistic effects between the odours of four lipid oxidation by-products (Venkateshwarlu *et al.*, 2004). Other sensory applications include wheat flour extrusions (Cheng & Fris, 2008) and orange beverages (Mirhosseini *et al.*, 2008; Mirhosseini *et al.*, 2009).

Performing sensory analysis on complex mixtures is limited and influenced by several factors. Firstly, there is a limit to the complexity of an odour that can accurately be profiled by judges (Lawless, 1999; Hughson & Boakes, 2002), which can be further limited by the number of compounds present (Livermore & Laing, 1996; Jinks, & Laing, 1999; Le Berre *et al.*, 2008a.) This situation is not improved by providing more information to judges (Jinks, & Laing, 1999). Furthermore, certain combinations of compounds enhance one another, whereas other combinations lead to suppressant effects (Laing, 1988; Laska & Hudson, 1991; Grosh, 2001; Anatosova *et al.*, 2005a; Anatosova *et al.*, 2005b). In addition, compounds have also been shown to interact differently at their detection thresholds compared to below their detection thresholds (Anatosova *et al.*, 2004). Blending effects also have a severe influence on the final odour character of a mixture (Brodin *et al.*, 2007; Le Berre *et al.*, 2008b). Another factor specifically influencing wine is that of semantic (linguistic) grouping; certain similar odours group together and are difficult to differentiate from one another if they form part of the same semantic group (Escudero *et al.*, 2007). These groups include similar specific odours (for example, blueberry, blackberry or strawberry) that all fall under a larger, more general descriptor like fruity, woody and sweet. Furthermore, effects found in intensity modelling in simple mixtures cannot be extrapolated to mixtures that are more complex (Brossard *et al.*, 2007).

In light of the above, the aim of this study was to profile combinations of 4-ethylphenol, 4-ethylguaiacol, 4-ethylcatechol and isovaleric acid in a young Pinotage red wine. The combinations were determined from a central composite design. The samples used for the central composite design were also presented to consumers in order to determine the consumer acceptance.

2 THEORY OF MULTIWAY METHODS

This section outlines the basic principles of the multiway method PARAllel FACtor analysis (PARAFAC), which has been applied in this paper. The outline is included due to novelty of

applying PARAFAC to sensory data, as examples in literature are limited. For other examples of the application of PARAFAC to sensory data, see Pravdova *et al.* (2002), Cocchi *et al.* (2006), Masino *et al.* (2008), and Bro *et al.* (2008).

Sensory data occur in a data cube (a three-way data matrix) as it contains one data table containing samples and attributes per judge. This can be seen in Figure 5.1. This data cube is usually simplified by unfolding the cube, and finding the means (Bro *et al*., 2008). However, the use of multiway models allow for a simpler interpretation of this kind of data. In this paper, PARAFAC will be used, but Tucker3 (a multiway method similar to PARAFAC) is referred to in aid of the explanation of the difference between PCA and PARAFAC.

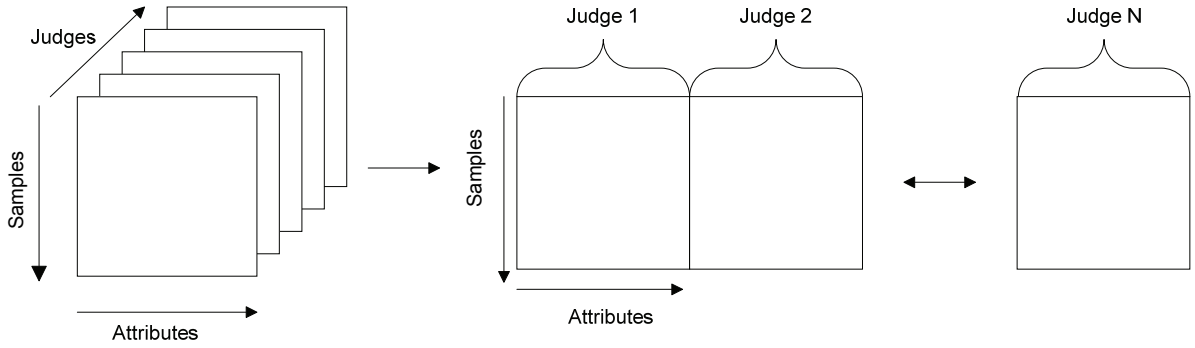
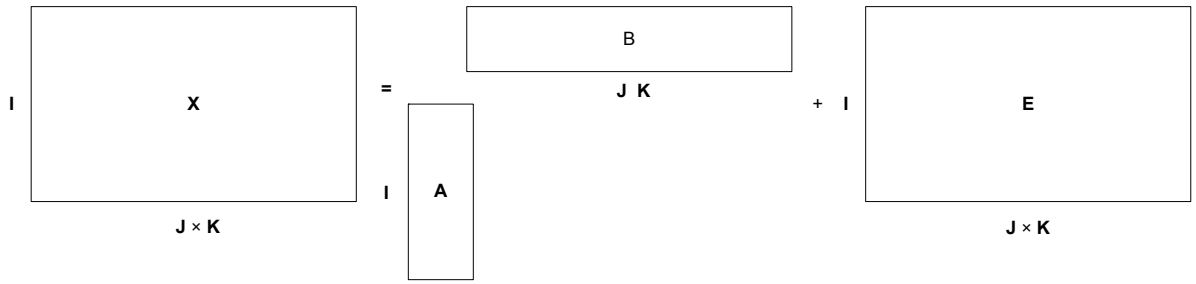


Figure 5.1. Structure of sensory data sets. Sensory data occur in a cube (Samples × Attributes × Judges) but is usually unfolded to form a two-way matrix.

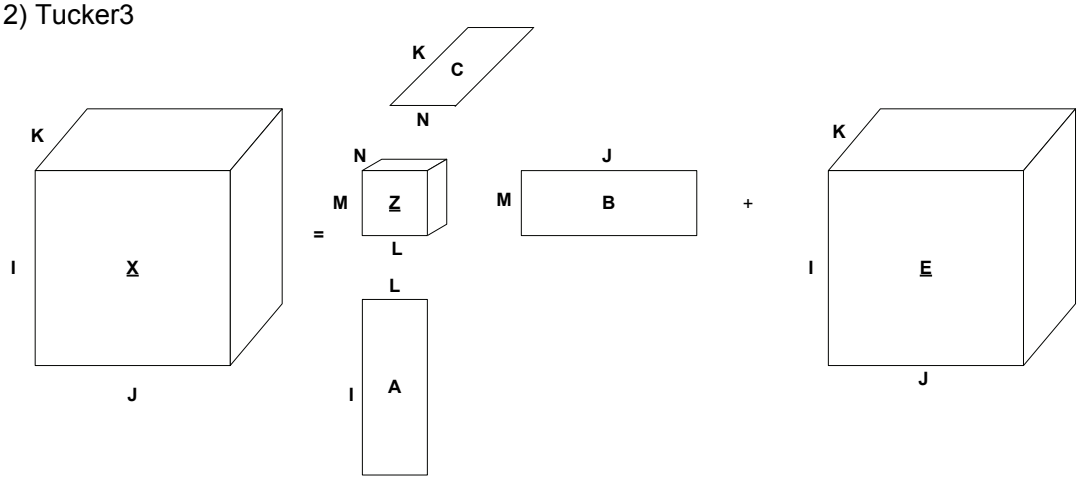
Figure 5.2 gives an outline of how PCA, Tucker3 and PARAFAC relate to one another. Tucker3 is a constrained version of PCA, whereas PARAFAC is a constrained version of the Tucker3 model. These constraints allow for the calculation of more robust and interpretable models, by preventing the model to include and model as much of the noise. Due to the constraint hierarchy, a two-way PCA model will always fit data better than a Tucker3 model, which will always fit data better than a PARAFAC model. However, PARAFAC focuses on modelling the systemic part of the data, because the final model has fewer parameters than a PCA model. This has the inherent implication that PARAFAC models are more easily interpreted than unfolded PCA models (Bro, 1997).

Apart from the fact that PCA is performed to a two-dimensional data matrix and PARAFAC is performed on a three-dimensional data array, the largest difference between PCA and PARAFAC is the way in which principal components or factors are calculated. In PCA, the components are calculated separately, and changing the number of components does not affect the model. The first principal component lies in the direction of the most variance, and subsequent orthogonal components are calculated to model the remainder of the variance.

1) PCA



2) Tucker3



3) PARAFAC

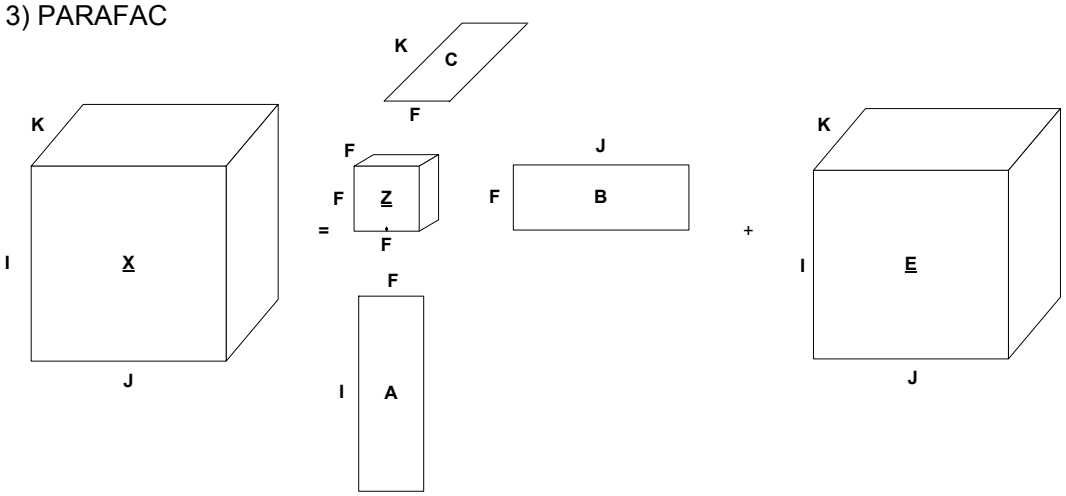


Figure 5.2. Graphical representation of unfolded PCA, Tucker3 and PARAFAC: 1) Unfolded PCA produces a scores matrix and a loading matrix (**A** and **B**) and a two-dimensional error matrix (**E**). 2) Tucker3 produces three loading matrices (**A**, **B**, and **C**), with a variable numbers of factors in each matrix (L, M and N). This results in a core array (**Z**) which can have variable proportions, corresponding to the number of factors in each loading matrix (L, M and N) and a three-dimensional error array (**E**). 3) PARAFAC also produces three loading matrices (**A**, **B** and **C**), which all contain the same number of factors (F). The resulting core array (**Z**) has equal proportions (F) in all three dimensions, and the error array (**E**) is three-dimensional.

The factors in PARAFAC are not constrained due to orthogonality, and are calculated simultaneously. These factors are calculated so that they collectively model the maximum amount of variance in the data. This is done by estimating two factors, calculating the remaining factors by least squares regression and recalculating the factors until convergence occurs. Convergence occurs when recalculating the factors no longer changes the model. This means that the number of factors in the model significantly affects the model itself, making the choice of number of factors of utmost importance (Bro, 1997).

Tucker3 differs from PARAFAC in that Tucker3 can have different numbers of factors for each of the three modes (sets of results), whereas PARAFAC has the constraint of having the same number of all factors for all modes. In the Tucker3 model, the core matrix, Z, (see Figure 5.2) is used to interpret these different loading matrices. The core array gives information about which interactions are significant, the magnitude of their significance and the nature of the interactions (positive or negative). A further constraint of the PARAFAC model is that the super diagonal elements of the core element have to be equal to 1 (or very close to 1). This means that the interactions between all the loadings vectors have to be of the same importance and in the same direction. This has the implication that PARAFAC can be interpreted in a similar manner to PCA. The core consistency test is a test evaluating whether these elements are in fact equal to 1, and can be used to evaluate whether the appropriate number of factors have been selected for a PARAFAC model (Pravdova *et al*., 2002).

3 MATERIALS AND METHODS

3.1 Central composite design

The current experimental procedure was conducted with a central composite design (Venkateshwarlu *et al.*, 2004). This design consists of five levels of up to six components, and can be used to test interactions between different factors. However, it differs from a factorial design as not all combinations of all factors are tested. It is recommended as an appropriate design where there is a limit to the number of experiments that can be performed, making it particularly suitable for sensory experiments (Esbensen, 2002). If n is the number of factors to be tested, the design has n^2 “cube points” and $2n$ “star points”, as well as a “centre point”, which is replicated. The simplest variation of the design, a two-factor central composite, is shown in Figure 5.3.

This design method was used in the current study to investigate the interaction between four different *Brettanomyces* related components. This gave rise to a design with eight star points, 16 cube points and one centre point, and therefore 25 samples in total. The compositions of the different samples will be discussed in the appropriate section.

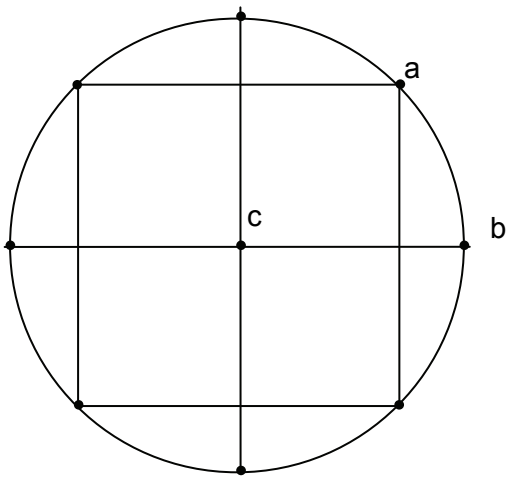


Figure 5.3. General layout of a two-factor central composite design. a) designates a cube point, b) designates a star point and c) designates the centre point.

3.2 Wine samples

Two hundred litres of Pinotage was locally obtained from wine producer (Distell Group Ltd, Stellenbosch, South Africa) during the course of 2009. This wine had been made using standard red wine making practices and completely underwent both alcoholic and malolactic fermentation and was bottled manually at the Department of Viticulture and Oenology, Stellenbosch University, South Africa. The wine used had an alcohol content of 11.6 %, and a pH of 3.65. This wine is identical to the one used in Chapter 4.

After bottling, the wine was analysed to determine the levels of 4-ethylphenol, 4-ethylguaiacol, 4-ethylcatechol and isovaleric acid present. The analyses for 4-ethylphenol, 4-ethylguaiacol and isovaleric acid were performed using GC-MS using a DB-FFAFF(60m x 320.0 µm x 0.5 µm) column, and the levels of 4-ethylcatechol were determined using HPLC-MS/MS using a C18 (2.1 x 50 mm with guard) column. All these analyses were conducted by an accredited laboratory (Quantum Laboratories, South Africa).

It was found that the base wine contained less than 10 µg/L all three volatile phenols investigated (4-ethylphenol, 4-ethylguaiacol and 4-ethylcatechol). The wine was therefore considered not to be spoiled by *Brettanomyces*. Furthermore, the wine contained 355 µg/L isovaleric acid. This value is in agreement with minimum values of this compound found in red wines (Francis & Newton, 2005).

3.3 Chemicals and spiking

Solutions of 4-ethylphenol, 4-ethylguaiacol, 4-ethylcatechol and isovaleric acid (Aldrich, South Africa) were prepared in 99.5% ethanol (Merck Chemicals, South Africa). Four solutions of each

compound was made, namely of 10 mg/mL, 1 mg/mL, 100 µg/mL and 10 µg/mL. These solutions were used to produce wine samples spiked with the exact concentrations of the latter compounds. The five levels used in this study were based on the central composite design, and were determined by the methodology outlined below.

The concentrations of the four different compounds to be used during this study were predetermined as follows: Firstly, detection thresholds were determined as described in Chapter 3. This detection level was set be approximately level 2 (the lower cube level, Figure 5.3) in the central composite design. This decision was made to allow for the investigation of sensory effects and interactions of the four compounds at, below, and above their detection thresholds. The highest level (level 5) corresponded to the highest level that is likely to occur naturally in wine. This level was determined with the guidance of literature, as well as with the help of wine-industry experts (Thales, South Africa). These levels were also subjected to extensive sensory pre-screening, to ensure that, in the wine used for this study, the concentrations conform to the sensory criteria set in t. These two levels were used to calculate the rest of the levels of the central composite design.

The central composite design levels were chosen along the same logarithmic scale as the eight levels used for the detection thresholds. These levels all satisfied the equation $c = ab^{n-1}$, where c is the concentration, a is the starting point (level 1), b is the multiplication factor and n is the level number. Levels chosen in the design were therefore on a simple numerical scale that corresponded to that used in the detection thresholds. This was done to allow to compensate for the fact that the distances between levels in the central composite design are predetermined. This created a difficulty as the highest levels that this research wished to investigate were between five and twenty times the magnitude of the detection threshold. The use of a logarithmic scale compensated for this difference in magnitude, and allowed for the concentrations in question to be fitted to the central composite design. For this reason, two sets of levels were used in this study. The "design" levels were those determined by the design, and fall between 0 and 13, and are shown in Table 5.2. The actual concentrations used were determined using the equation $c = ab^{n-1}$ and are shown in Table 5.1. In both these tables, levels are numbered 1 to 5, and these values are used throughout this chapter when referring to samples, with reference to the concentrations and design levels where necessary.

Table 5.1. Actual concentrations of Brett-related compounds tested (µg/L).

	Level				
Compound	**1**	**2**	**3**	**4**	**5**
4-ethylphenol	82	227	623	1711	4695
4-ethylguaiacol	65	117	230	381	688
4-ethylcatechol	181	290	465	745	1193
Isovaleric acid	381	431	577	997	2210

The final concentration ranges were confirmed with the help of wine-industry experts (Thales, South Africa) by means of several sessions of consensus sensory analysis. Before commencement of the sensory tests, the final samples were also subjected to sensory pre-assessment.

Table 5.2. Design levels for spiking of wines with Brett-related compounds (arbitrary scale).

Compound	Level				
	1	2	3	4	5
4-ethylphenol	0.5	3.5	6.5	9.5	12.5
4-ethylguaiacol	0.75	2.5	4.5	6	7.75
4-ethylcatechol	2.8	4.2	5.6	7	8.4
Isovaleric acid	2.5	4.5	6.5	8.5	10.5

The final sample set is shown in Table 5.3. Please note that throughout this document, samples will be referred to by means of four-digit codes. These codes correspond to the levels used in Table 5.1 and Table 5.3, and are listed in the order 4-ethylphenol, 4-ethylguaiacol, 4-ethylcatechol, isovaleric acid. For example, sample 2442 contains level 2 (227 µg/L) of 4-ethylphenol, level 4 (381 µg/L) of 4-ethylguaiacol, level 4 (745 µg/L) of 4-ethylcatechol and level 2 (431 µg/L) of isovaleric acid.

3.4 Profiling of central composite design combination samples

The combination samples, shown in Table 5.3, were profiled after the descriptive analysis of the singular compounds had been completed. The sensory panel used consisted of 10 judges, of whom seven participated in a study involving the profiling of the compounds in this study, and therefore had experience in analysis for Brett character. These judges were therefore already familiar with the aroma profiles associated with the compounds used in this study.

Training commenced with three sessions of general training, which allowed the judges to familiarise themselves with the samples in question. These samples were presented in three different subsets, which were analysed on three different days. The first subset was the eight star samples, the second subset the first eight cube samples (cube 1 – cube 8), and the third subset the final 8 cube samples (cube 9 – cube 16). During all three training sessions, the reference standards listed in Table 5.4 were made available to the judges. Additionally, the centre sample, as well as the highest levels of the four individual compounds (level 5) was presented to the judges to serve as reference standards. During training, all samples were labelled using a system that indicated their composition to the judges.

Table 5.3. Compositions of the different samples in the design.

	Compound			
Sample	**4-ethylphenol**	**4-ethylguaiacol**	**4-ethylcatechol**	**Isovaleric acid**
Centre	3	3	3	3
Star 1	5	3	3	3
Star 2	1	3	3	3
Star 3	3	5	3	3
Star 4	3	1	3	3
Star 5	3	3	5	3
Star 6	3	3	1	3
Star 7	3	3	3	5
Star 8	3	3	3	1
Cube 1	4	4	4	4
Cube 2	4	4	4	2
Cube 3	4	4	2	4
Cube 4	4	4	2	2
Cube 5	4	2	4	4
Cube 6	4	2	4	2
Cube 7	4	2	2	4
Cube 8	4	2	2	2
Cube 9	2	4	4	4
Cube 10	2	4	4	2
Cube 11	2	4	2	4
Cube 12	2	4	2	2
Cube 13	2	2	4	4
Cube 14	2	2	4	2
Cube 15	2	2	2	4
Cube 16	2	2	2	1

The general training was followed with three subsequent training sessions. During each of these sessions, one of the three sets used during general training was presented to the judges, and the samples were scaled on a 100 mm unstructured line scale according to the attributes in Table 5.4. The reference standards listed in Table 5.4 were adapted from Noble *et al.* (1987) and used during a previous study (Chapter 4). Note that the descriptors used in this study differ slightly from those used in Chapter 4. The number of descriptors were reduced as the panel could not accurately perceive all the descriptors used in the samples containing the individual compounds in the combination samples. An example of such a descriptor is the leather like attribute used in Chapter 4. In the samples in Chapter (4-ethylphenol) where this descriptor was relevant, this descriptor could be distinguished by the panel from the Elastoplast™ descriptor. However, no such distinction could be made in the combination samples.

The final profiling analyses were conducted by 10 trained assessors in booths with standard artificial daylight lighting and temperature control at 20°C ±1°C. The wine was analysed in standard ISO wine tasting glasses, sample size was 20 mL and samples were

served at 20°C ±1°C. Prior to the analysis, the wine glasses containing the wine samples were covered with plastic lids. This prevented the aroma of the wine from escaping or contaminating the laboratory environment.

The analyses were performed as six separate tests with two replications. During each test, the judges received five samples for analysis, which included the centre sample and four other samples from the design. The samples for analysis were labelled with a random three-digit code and were presented in a randomised order. The judges also received the non-spiked control wine, as well as a wine sample containing the highest concentration (level 5) of each compound. The first two tests contained the star samples, and the last four tests contained the cube samples. This division was selected based on the recommendation by Esbensen (2002). However, the samples were randomised between tests and judges.

Table 5.4. Descriptors and reference standards used during descriptive analysis of wines spiked with a combination of four Brett-related compounds. Note that two reference standards were presented for the smoky-savoury attribute and that the panel was instructed to form a cognitive combination between these two attributes.

Descriptor	Definition	Reference standard
Berry-like	Typical wine-sweet berry-like aroma	Control sample
Sick-sweet	Atypical wine-sweet – sweet smell that is uncharacteristic/ unnatural for wine (confected)	None
Elastoplast™	Smell associated with Elastoplast™ or Band-Aid™	1 piece of Elastoplast™ fabric bandage
Medicinal (Listerine)	Minty smell associated with mouthwash	Wine spiked with Listerine™ (3 drops in 100 mL wine)
Smoky-savoury	The smell associated with smoked food	A cognitive combination between smoke essence (3 drops in 100 mL wine) and soy sauce (1 ml in 100 mL wine)
Pungent	Sweaty/ rancid/ cheesy/ vinegary	A small piece of mild blue cheese (Simonzola™)

3.5 Consumer analysis

A consumer analysis was performed using 100 consumers sourced from the Stellenbosch area. Fifty percent of the consumers indicated that they enjoyed drinking wine but did not know much about it, whereas the remaining fifty percent indicated that they knew a fair amount about wine. The consumer analysis was performed using an incomplete block design; where each consumer received a set of five of the spiked samples and an unspiked (control) sample. All the samples were labelled with a random three-digit code and were presented in a random order. Questions were also posed about the wine consumption patterns of the consumers, as well as

their degree of wine expertise. Biographical data (gender, age) were obtained for each individual consumer.

The consumers were instructed to smell and taste the wine samples in the order presented. Each consumer received a water biscuit (Carr, UK) and water to clean their palate before and after tasting each sample. The consumers had to indicate their degree of liking of the samples on a standard nine-point hedonic scale where 1 represents *Dislike extremely* and 9 represents *Like extremely* (Lawless & Heymann, 1998). In order to prevent the interference effect of information regarding wine quality on hedonic ratings (Sigrist & Cousin, 2009), no information was provided regarding the purpose of test.

3.6 Data analysis

3.6.1 Central composite design

The data were analysed using SAS® software (Version 9; SAS Institute Inc, Cary, USA) and subjected to the Shapiro-Wilk test for non-normality of the residuals (Shapiro & Wilk, 1965). If non-normality was found to be significant ($P \leq 0.05$) and caused by skewness, the outliers were identified and removed until the data were normal or symmetrically distributed (Glass *et al.,* 1972). Using line plots indicating temporal stability and internal consistency, single odd judges were identified and removed. The final analysis of variance (ANOVA) was performed after all the above-mentioned procedures have taken place. Student's t-least significant difference (LSD) was calculated at the 5% significance level to compare treatment means.

If second–order interactions between compounds for specific descriptors were found to be significant, a curve was fitted between the means of these compounds using the TableCurve 3D software (Version 4.0, SYSTAT Software Inc). If third-order interactions were found to be significant, the ANOVA was repeated with one of the compounds in the interaction kept as a constant. The results of these analyses were interpreted in a similar manner to those of the original analyses.

PCA was performed on the mean values of the overall profiles of the wines. Furthermore, Partial Least Squares regression (PLS) was performed using the chemical information in the X-space and the sensory descriptive data in the Y-space. During this analysis, the chemical data was converted to category variables, in order to investigate the difference in effect of the different levels of the compounds. Both these multivariate techniques were performed using the XLStat software package (Version 2009.5.0.1, Addinsoft, SARL, Paris, France).

PARAFAC was performed in Matlab (Mathworks, Inc) using the PLS toolbox version 5.3 (EigenVector Research Inc, Manson, WA, US). PARAFAC was performed on the same dataset as the ANOVA, i.e. the raw scores produced by judges tested for outliers.

3.6.2 Consumer panel

The unspiked sample was considered as a *standard sample* for all the consumers and therefore the data were pooled for analysis of variance (ANOVA) (SAS®, Version 9; SAS® Institute Inc, Cary, USA.). The Shapiro-Wilk test was used to test for non-normality of the residuals (Shapiro & Wilk, 1965). If skewness appeared to be the result of outliers these outliers were identified and discarded until the data were considered normal or symmetrically distributed (Glass *et al.,* 1972). Furthermore, two types of preference mapping were performed. Firstly, external preference mapping was performed by superimposing the consumer data on the PCA map of the sensory profiles. Secondly, PLS was performed with the sensory data in the X-space and the consumer data in the Y-space. Both these techniques were performed using the software package XLStat (Version 2009.5.0.1, Addinsoft, SARL, Paris, France).

4 RESULTS AND DISCUSSION

4.1 Profiling of samples

Please note that in this section, occasional reference is made to results obtained during singular profiling of these four compounds. These results are discussed in Chapter 4, and are not included in this section for the sake of simplicity.

4.1.1 Berry-like

The ANOVA for the berry-like attribute is shown in Table 5.5. As can be seen in this table, a significant interaction ($p = 0.0357$) was found between the level of 4-ethylphenol and the level of 4-ethylcatechol. This interaction was plotted and a curve with the equation $z = 233.89 + 0.159\ln x + 0.116 (\ln x)^2 + 0.412(\ln x)^3 + 183.96y + 54.03y^2 + 6.70y^3 + 0.29y^4$ was obtained. This function has a coefficient of determination of $R^2 = 0.52$ and is shown in Figure 5.4. This is not considered a particularly good fit, but in spite of this, several general trends can be observed from the curve.

Table 5.5. ANOVA performed on sensory data obtained for the berry-like attribute.

Source	DF	Mean Square	Pr > F
4-EP	4	797	0.0001
4-EG	3	228	0.033
4-EC	3	59.9	0.51
ISOV	3	338	0.005
4-EP*4-EG	1	0.58	0.93
4-EP*4-EC	1	344	0.036
4-EG*4-EC	1	114	0.22
4-EP*ISOV	1	0.64	0.93
4-EG*ISOV	1	207	0.10
4-EC*ISOV	1	72.8	0.33
4-EP*4-EG*4-EC	1	76.5	0.32
4-EP*4-EG*ISOV	1	0.021	0.99
4-EP*4-EC*ISOV	1	4.75	0.80
4-EG*4-EC*ISOV	1	8.34	0.74
4-EP*4-EG*4-EC*ISOV	1	119	0.22
Error	495	77.6	

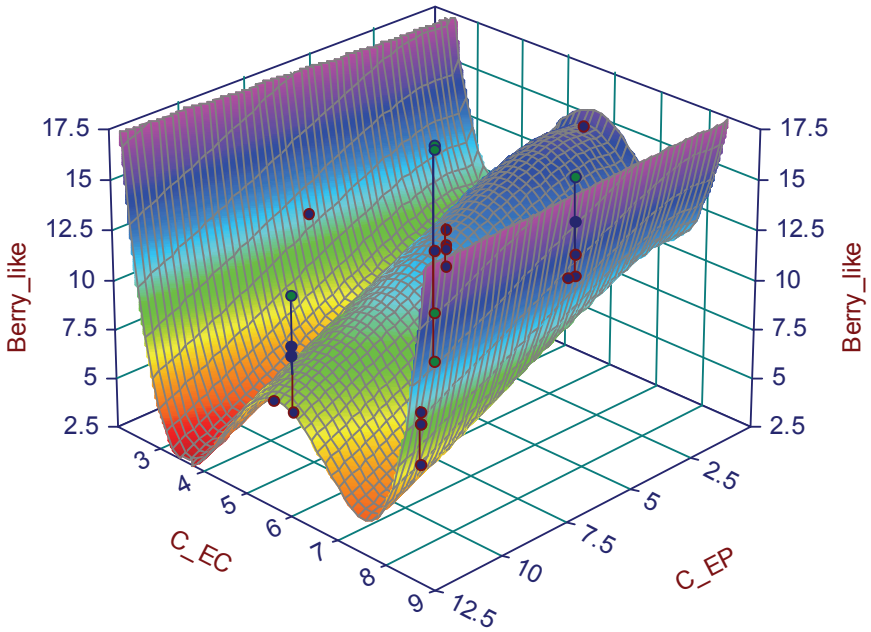


Figure 5.4. Interaction between 4-ethylphenol and 4-ethylcatechol in terms of berry-like character in Pinotage red wine. Note that the figure shows the highest values in the front, in order for the curve to be visible. C_EC refers to the "design" level of 4-ethylcatechol, and C_EP refers to that of 4-ethylphenol.

The berry-like attribute firstly generally decreased with an increase in 4-ethylphenol, as the curve slopes upwards in the direction of decreasing 4-ethylphenol concentration. 4-ethylcatechol, however, appears to have a "wavy" effect associated with it, in other words –

higher berry-like character at odd levels than at even levels, with the highest berry-like character found at the highest and lowest levels. At first, this may appear to be strange, but this pattern could be explained by considering the design.

At the highest and lowest levels of 4-ethylcatechol, all other compounds are at level 3, and the berry-like suppressant effect is the least. However, when 4-ethylcatechol is at level 3 (design level 5.6), it is combined with the lowest or highest levels of the other compounds, which all have been shown to a have a suppressant effect on berry-like character. In addition, when 4-ethylcatechol is at levels 2 (design level 4.2) and 4 (design level 7), it is combined with several other levels of all the other compounds, some combinations containing all the compounds at their highest levels, which may have the highest suppressant effect on the berry-like attribute. Therefore, it can be concluded that 4-ethylcatechol had very little suppressant effect on the berry-like character, and that its observed interaction with 4-ethylphenol is most probably an artefact of the central composite design.

As is also evident from Table 5.5, the effect of isovaleric acid can be interpreted as the only significant main effect ($p = 0.0048$). The effect of an increase in isovaleric acid is shown in Figure 5.5 and Table 5.6. As can be seen from both this table and this figure, there is a general decrease in the level of berry-like character with an increase in concentration of isovaleric acid. Level 1 (381 µg/L) and level 3 (577 µg/L) are significantly different, and level 3 and level 5 (2210 µg/L) are also significantly different. However, there is no significant difference between level 2 (577 µg/L) and 4 (997 µg/L) and either level 3 (577 µg/L) or 5 (2210 µg/L). This is probably because when isovaleric acid is at levels 2 and 4 the other compounds are all either at level 2 or 4. This means that the lower values for berry-like character observed at level 2 and 4 of isovaleric acid may in fact be due to the combined suppression of berry-like character by the higher levels (level 4) of the other compounds. However, the overall trend of a drop in berry-like character is similar to that observed in all the compounds when profiled on their own. An important difference, however, is the fact that the suppression occurs over a different range than when isovaleric acid in profiled in its singular state. The suppression of berry-like character occurs from 34 mm on the 100 mm scale at level 1 to 26 mm on the 100 mm scale at level 5 when only isovaleric acid is added to the wine but from 16 mm at level 1 to 6 mm at level 5 when in combination with the other compounds. This further indicates that there is a synergistic effect in the suppression of berry-like character between the different compounds.

Although the suppression of berry-like character by 4-ethylguaiacol is evident in Chapter 4, no significant effect or significant interactions were observed in Table 5.5, and the compound is therefore not discussed.

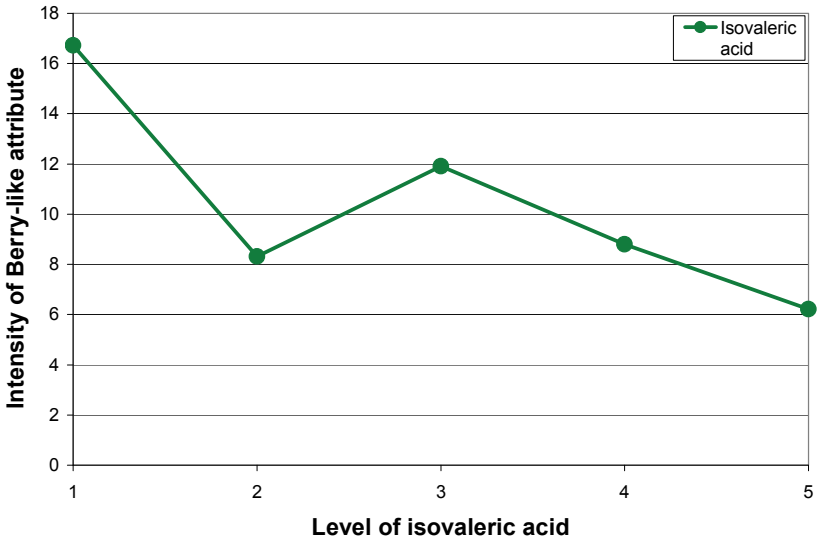


Figure 5.5. Effect of isovaleric acid on berry-like character during combination profiling in Pinotage red wine.

Table 5.6. Least significant difference groups of isovaleric acid on berry-like character profiling in Pinotage red wine.

Level	Mean[1]
1	16.7 [a]
2	8.8 [b c]
3	11.9 [b]
4	8.8 [b c]
5	6.2 [c]
Least Significant Difference (p = 0.05)	**3.94**

[1] Values with the same superscript are not significantly different

4.1.2 Sick-sweet

The results of the ANOVA for the sick-sweet characteristic are shown in Table 5.7. As shown in this table, a significant interaction (p = 0.0302) was found between 4-ethylcatechol and 4-ethylphenol. Although this interaction was significant, no curve could be fitted that had an R^2 value of greater than 0.5. For this reason, this interaction was investigated manually. The effect of 4-ethylphenol on 4-ethylcatechol is shown in Figure 5.6, and the effect of 4-ethylcatechol on 4-ethylphenol is shown in Figure 5.7. The effects are summarised in Table 5.8. In Table 5.8, an overall change in sick-sweet of less than one unit was taken to be no change.

As can be seen in Figure 5.6, Figure 5.7 and Table 5.8, both these compounds prevented an increase in sick-sweet character by the other compound when they were at level 2 or level 3 (therefore at detection threshold or just above detection threshold). This means that both these compounds interfered with the sick-sweet effect of each other at these levels. At

level 4, both compounds allowed a change in sick-sweet character to occur with an increase in the other compound. However, none of these increases were severe enough that the effect could be described as synergistic.

Table 5.7. ANOVA performed on the sensory data obtained for the sick-sweet attribute.

Source	DF	Mean Square	Pr>F
4-EP	4	73.1	0.051
4-EG	3	101	0.021
4-EC	3	26.6	0.45
ISOV	3	5.53	0.91
4-EP*4-EG	1	22.8	0.38
4-EP*4-EC	1	145	0.030
4-EG*4-EC	1	1.23	0.84
4-EP*ISOV	1	0.455	0.90
4-EG*ISOV	1	0.451	0.90
4-EC*ISOV	1	28.4	0.34
4-EP*4-EG*4-EC	1	19.4	0.43
4-EP*4-EG*ISOV	1	4.15	0.71
4-EP*4-EC*ISOV	1	0.442	0.91
4-EG*4-EC*ISOV	1	65.6	0.15
4-EP*4-EG*4-EC*ISOV	1	13.2	0.51
Error	493	30.7	

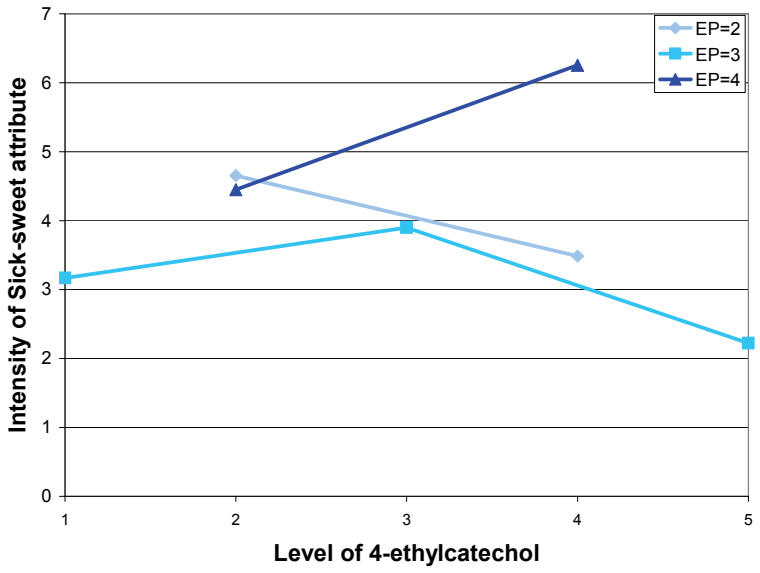


Figure 5.6. Effect of different levels of 4-ethylphenol on the sick-sweet character caused by 4-ethylcatechol in Pinotage red wine.

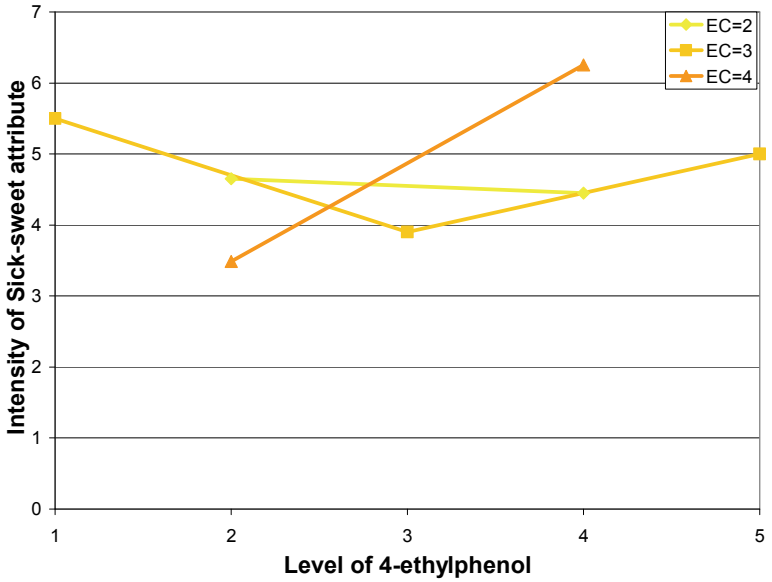


Figure 5.7. Effect of different levels of 4-ethylcatechol on the sick-sweet character caused by 4-ethylphenol in Pinotage red wine.

Table 5.8. Summary of the effects of 4-ethylphenol and 4-ethylcatechol in combination on sick-sweet character in Pinotage red wine.

Compound	Level	Effect	Expected[a]
4-ethylphenol	2	Sick-sweet stays constant	No
	3	Sick-sweet stays constant	No
	4	Sick-sweet increases with an increase with 4-ethylcatechol	Yes
4-ethylcatechol	2	Sick-sweet stays constant with increase of 4-ethylphenol	No
	3	Sick-sweet stays constant	No
	4	Sick-sweet increases with an increase with 4-ethylphenol	Yes

[a] Expected according to previous results (Chapter 4)

Table 5.9. Change in the sick-sweet character with level of 4-ethylguaiacol in Pinotage red wine.

Level	Mean[1]
1	2.6 [b]
2	3.8 [a b]
3	4.1 [a b]
4	5.7 [a]
5	3.3 [a b]
Least Significant Difference (p = 0.05)	**2.48**

[1] Values with the same superscript are not significantly different

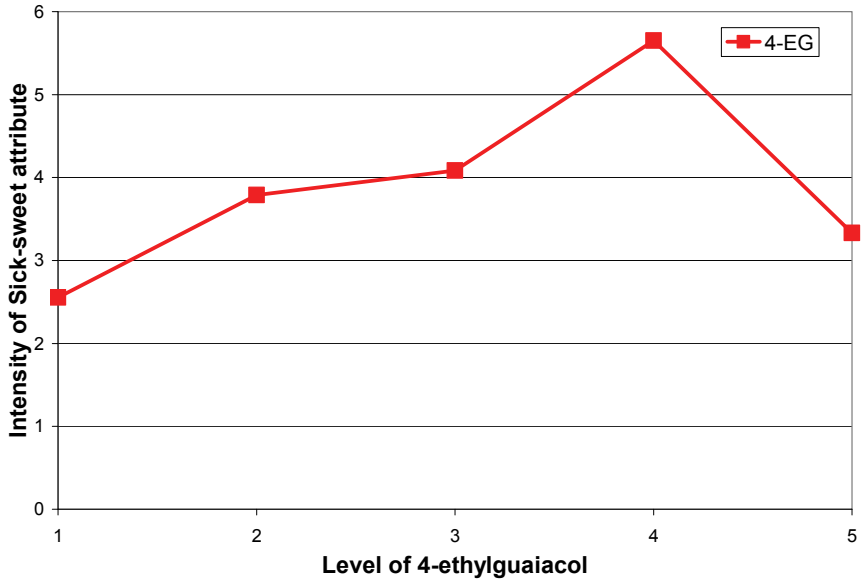


Figure 5.8. Effect of 4-ethylguaiacol on sick-sweet character in Pinotage red wine in combination samples.

The only main statistical effect that could be interpreted was that of 4-ethylguaiacol (Table 5.7). The change in sick-sweet character with the increase in 4-ethylguaiacol is shown in Table 5.9 and Figure 5.8

Figure 5.8 and Table 5.9 show an unexpected trend in terms of sick-sweet character. The means for levels 1 and 4 differed significantly from one another, but none of the other means differed significantly from either level 1 or level 4. This may be because the greatest synergistic effect in terms of sick-sweet happens at level 4 of 4-ethylguaiacol. It is, however, interesting to note that the sample with the highest mean for sick-sweet was the one that contained level 4 of all four the compounds. This mean is 7.72, which was more than one unit higher than its closest successor. It could be that the synergistic effect between the different compounds in terms of sick-sweet character has caused this.

It is finally interesting to note that the overall increase in sick-sweet character was extremely small – from 2 to about 6, which may be due to several reasons. Firstly, the sick-sweet characteristic is one that the panel found particularly difficult to define, and some panel members had difficulty picking up this characteristic in the combination samples. This is not unlike what has been reported in literature: that the performance of panel members decrease with complexity of samples (Hughson & Boakes, 2002). For this reason, this difficult-to- perceive characteristic could not be picked up above the other aromas being profiled. Another possibility is the fact that the sick-sweet attribute (which becomes perceptible due to the suppression of the natural berry-like character in the wine) is not perceptible in samples that contain a combination of these Brett-related compounds (and therefore in wines spoiled by *Brettanomyces*). This is particularly plausible, as, to date, the term “sick-sweet” has not been coupled with wines naturally spoiled by *Brettanomyces*. However, the descriptor was included in

this study as it was used in Chapter 4, and was distinctly perceived in the samples that contained the individual compounds.

4.1.3 Elastoplast™

The ANOVA for the Elastoplast™ attribute, as seen in Table 5.10, showed two significant three-way interactions: firstly between 4-ethylphenol, 4-ethylguaiacol and 4-ethylcatechol ($p = 0.0062$), and secondly between 4-ethylphenol, 4-ethylguaiacol and isovaleric acid ($p = 0.0253$). This indicates that 4-ethylphenol interacts significantly with three the other three compounds in term of the Elastoplast™ descriptor. As these three-way interactions cannot be directly interpreted, ANOVA's were performed at constant levels of 4-ethylcatechol and isovaleric acid in order to investigate the interaction between 4-ethylphenol and 4-ethylguaiacol. Results for these analyses are shown in Table 5.11, Table 5.12, Table 5.13 and Table 5.14.

Table 5.10. ANOVA performed on the sensory data obtained for the Elastoplast™ attribute.

Source	DF	Mean Square	Pr>F
4-EP	4	4502	0.0001
4-EG	3	72.6	0.39
4-EC	3	131	0.14
ISOV	3	15.6	0.89
4-EP*4-EG	1	272	0.052
4-EP*4-EC	1	171	0.12
4-EG*4-EC	1	94.2	0.25
4-EP*ISOV	1	50.0	0.41
4-EG*ISOV	1	427	0.015
4-EC*ISOV	1	209	0.089
4-EP*4-EG*4-EC	1	544	0.006
4-EP*4-EG*ISOV	1	363	0.025
4-EP*4-EC*ISOV	1	31.2	0.51
4-EG*4-EC*ISOV	1	12.3	0.68
4-EP*4-EG*4-EC*ISOV	1	10.4	0.71
Error	491	72.1	

Table 5.11. ANOVA of 4-ethylphenol and 4-ethylguaiacol at level 2 of 4-ethylcatechol in Pinotage red wine.

Source	DF	Mean Square	Pr>F
4-EP	1	787	0.0009
4-EG	1	95.7	0.24
4-EP*4-EG	1	23.4	0.56
Error	121	68.4	

Table 5.12. ANOVA of 4-ethylphenol and 4-ethylguaiacol at level 4 of 4-ethylcatechol in Pinotage red wine.

Source	DF	Mean Square	Pr>F
4-EP	1	2158	0.0001
4-EG	1	17.3	0.65
4-EP*4-EG	1	809	0.002
Error	122	81.5	

Table 5.13. ANOVA of 4-ethylphenol and 4-ethylguaiacol at level 2 of isovaleric acid in Pinotage.

Source	DF	Mean Square	Pr>F
4-EP	1	1055	0.0004
4-EG	1	301	0.052
4-EP*4-EG	1	3.11	0.84
Error	122	78.3	

Table 5.14. ANOVA of 4-ethylphenol and 4-ethylguaiacol at level 4 of isovaleric acid in Pinotage.

Source	DF	Mean Square	Pr>F
4-EP	1	1753	0.0001
4-EG	1	134	0.19
4-EP*4-EG	1	656	0.004
Error	121	76.5	

It was found that there was no significant interaction between 4-ethylphenol and 4-ethylguaiacol at level 2 of either 4-ethylcatechol or isovaleric acid (see Table 5.11 and Table 5.13). However, there were significant interactions at level 4 of 4-ethylcatechol ($p = 0.0020$) (see Table 5.12) and at level 4 of isovaleric acid ($p = 0.0041$) (see Table 5.14), which are shown in Figure 5.9 and Figure 5.10.

The graph in Figure 5.9 was fitted to the an equation of $z = 20.09 - 4.222 \ln x - 112.33 / y + 0.1364 (\ln x)^2 + 77.52 / y^2 + 40.82 (\ln x)/y$ (R^2 value of 0.8126). It can be seen that at level 2 of 4-ethylcatechol, 4-ethylguaiacol appears to enhance the Elastoplast™ effect associated with 4-ethylphenol with an increase in concentration. However, at level 4, 4-ethylguaiacol appears to suppress the Elastoplast™ descriptor with an increase in concentration. This effect may be due to a third-order interaction occurring at this level. Figure 5.10 ($z = 2{,}61 + 1{,}03x – 15{,}46/y + 0{,}1200x^2 + 26{,}81/y^2 + 6{,}03\ x/y$, $R^2 = 0.8433$) shows a similar overall effect. Both these effects justify doing more two-way ANOVA's at constant levels of 4-ethylguaiacol and therefore investigating the interactions of 4-ethylcatechol and isovaleric acid with 4-ethylphenol. The results of these ANOVA's are shown in Table 5.15, Table 5.16, Table 5.17 and Table 5.18.

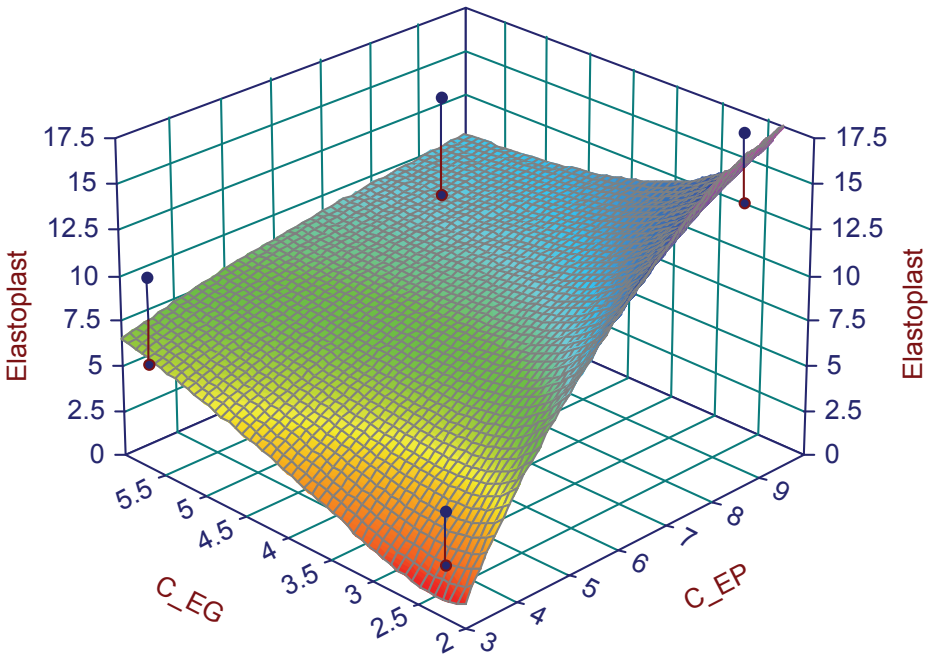


Figure 5.9. Effect of 4-ethylphenol and 4-ethylguaiacol on the Elastoplast™ descriptor at level 4 of 4-ethylcatechol. C_EG designates the design level of 4-ethylguaiacol, and C_EP indicates the design level of 4-ethylphenol.

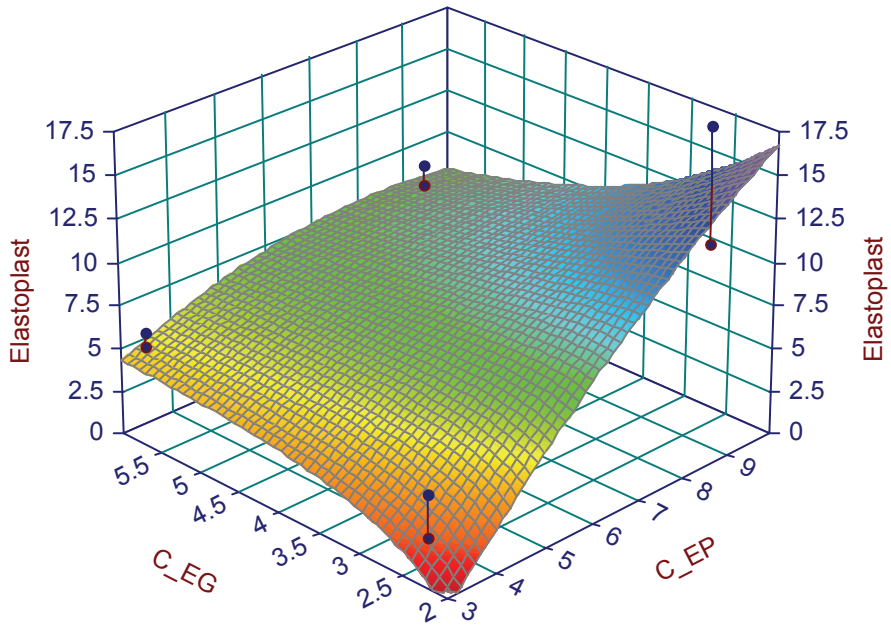


Figure 5.10. Interaction between 4-ethylguaiacol and 4-ethylphenol on the Elastoplast™ descriptor at isovaleric acid level 4. C_EG designates the design level of 4-ethylguaiacol, and C_EP indicates the design level of 4-ethylphenol.

Table 5.15. ANOVA on the effect of 4-ethylphenol and 4-ethylcatechol on Elastoplast™ when 4-ethylguaiacol is at level 2.

Source	DF	Mean Square	Pr>F
4-EP	1	2369	<.0001
4-EC	1	411	0.024
4-EP*4-EC	1	657	0.005
Error	122	79.0	

Table 5.16. ANOVA on the effect of 4-ethylphenol and 4-ethylcatechol on Elastoplast™ when 4-ethylguaiacol is at level 4.

Source	DF	Mean Square	Pr>F
4-EP	1	622	0.0042
4-EC	1	42.9	0.44
4-EP*4-EC	1	47.1	0.42
Error	121	73.0	

Table 5.17. ANOVA on the effect of 4-ethylphenol and isovaleric acid on Elastoplast™ when 4-ethylguaiacol is at level 2.

Source	DF	Mean Square	Pr>F
4-EP	1	2370	0.0001
ISOV	1	102	0.27
4-EP*ISOV	1	325	0.052
Error	122	84.3	

Table 5.18. ANOVA on the effect of 4-ethylphenol and isovaleric acid on Elastoplast™ when 4-ethylguaiacol is at level 4.

Source	DF	Mean Square	Pr>F
4-EP	1	622	0.0036
ISOV	1	328	0.032
4-EP*ISOV	1	76.3	0.30
Error	121	70.4	

When ANOVA's were performed at constant levels of 4-ethylguaiacol, it was found that there was a significant interaction ($p = 0.0518$) between 4-ethylphenol and isovaleric acid at 4-ethylguaiacol level 2 (see Table 5.17), as well as between 4-ethylphenol and 4-ethylcatechol ($p = 0.0046$) (see Table 5.15). These interactions are shown in Figure 5.11 ($Z = 1.25 + 0.135x + 45.7/y + 0.31x^2 + 42.8/y^2 + 15.1\ x/y$, $R^2 = 0.87$) and Figure 5.12 ($Z = 10.76 – 2.07\ln x – 11.66\ln y - 2.09(\ln x)^2 – 0.69(\ln y)^2 + 9.68\ln x\ln y$, $R^2 = 0.71$.)

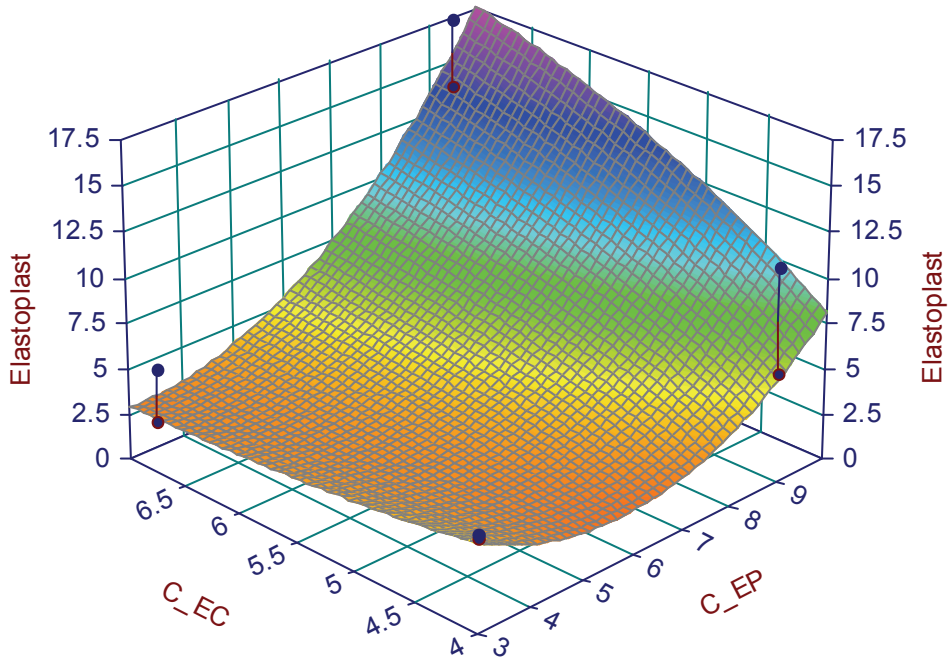


Figure 5.11. Interaction between 4-ethylphenol and 4-ethylcatechol for Elastoplast™ descriptor at 4-ethylguaiacol level 2. C_EC designates the design level of 4-ethylcatechol, and C_EP indicates the design level of 4-ethylphenol.

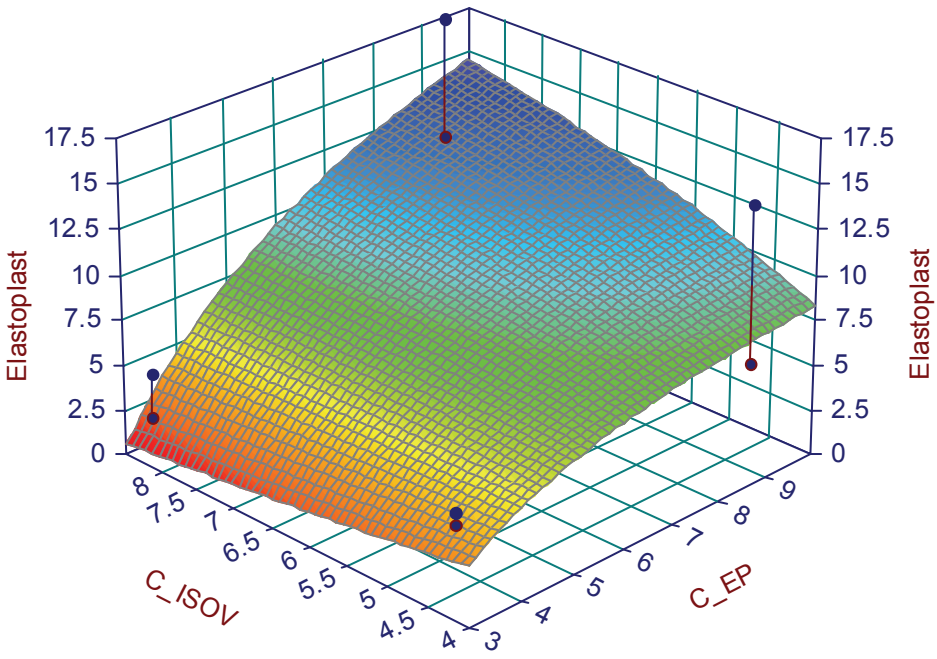


Figure 5.12. Interaction between isovaleric acid and 4-ethylphenol for Elastoplast™ descriptor for 4-ethylguaiacol level 2. C_ISOV designates the design level of isovaleric acid, and C_EP indicates the design level of 4-ethylphenol.

As can be seen in these figures, both 4-ethylcatechol and isovaleric acid showed synergistic effects with 4-ethylphenol in terms of the Elastoplast™ characteristic when 4-

ethylguaiacol was at level 2. The Elastoplast™ effect of 4-ethylphenol increased with an increase in 4-ethylcatechol, as well as with an increase in isovaleric acid. However, this effect is more severe in the case of 4-ethylcatechol than in the case of isovaleric acid.

4.1.4 Medicinal

The results of the ANOVA for the medicinal attribute are shown in Table 5.19. There were no significant interactions for the medicinal descriptor, but three of the compounds, namely 4-ethylguaiacol ($p = 0.0001$), 4-ethylcatechol ($p = 0.0365$) and isovaleric acid ($p = 0.0410$) could be identified as statistical main effects. The means of these compounds are shown in Table 5.20 and Figure 5.13.

Table 5.19. Results of the ANOVA performed on the sensory data obtained for the medicinal attribute.

Source	DF	Mean Square	Pr > F
4-EP	4	58.0	0.398
4-EG	3	702	0.0001
4-EC	3	163	0.037
ISOV	3	158	0.041
4-EP*4-EG	1	16.3	0.59
4-EP*4-EC	1	0.15	0.96
4-EG*4-EC	1	5.22	0.76
4-EP*ISOV	1	0.056	0.98
4-EG*ISOV	1	18.0	0.57
4-EC*ISOV	1	23.5	0.52
4-EP*4-EG*4-EC	1	59.9	0.31
4-EP*4-EG*ISOV	1	1.10	0.89
4-EP*4-EC*ISOV	1	22.6	0.53
4-EG*4-EC*ISOV	1	19.7	0.56
4-EP*4-EG*4-EC*ISOV	1	16.8	0.59
Error	495	57.1	

As expected, the medicinal attribute increased with an increase in the level of 4-ethylguaiacol. There was a significant difference between levels 1 and 4, levels 2 and 4 and between levels 4 and 5. It is interesting to note that the means for 4-ethylguaiacol and medicinal followed almost exactly the same pattern as the means obtained for 4-ethylguaiacol during the singular profiling. The range and the means were also similar, as the singular mean for level 1 was 5.5 and 17.2 for level 5. However, the values obtained during combination profiling were consistently lower than those obtained during singular profiling.

Table 5.20. Means for changes in medicinal attribute in Pinotage red wine with changes in concentration of different compounds.

Level	Mean 4-ethylguaiacol	Mean 4-ethylcatechol	Mean Isovaleric acid
1	4.3 [c]	11.8 [a]	9.9 [a]
2	5.3 [c]	7.1 [b]	7.4 [a]
3	6.9 [b c]	7.1 [b]	7.5 [a]
4	8.8 [b]	7.0 [b]	6.7 [a]
5	14.9 [a]	5.1 [b]	3.0 [b]
Least Significant Difference (p = 0.05)	**3.38**	**3.38**	**3.38**

[1] Values with the same superscript are not significantly different

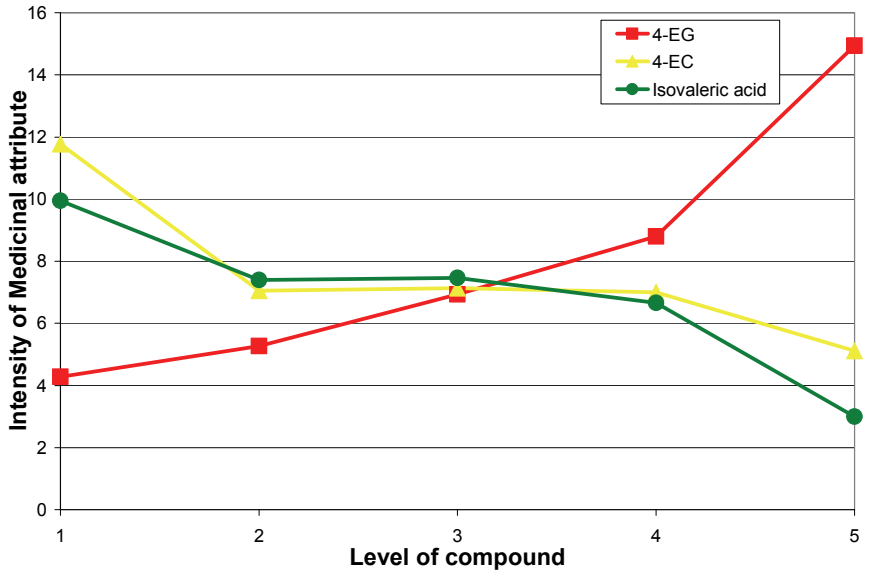


Figure 5.13. Change in medicinal attribute with different levels of 4-ethylguaiacol, 4-ethylcatechol and isovaleric acid.

The patterns for 4-ethylcatechol and isovaleric acid were the opposite of that found with 4-ethylguaiacol. Here the means consistently decreased with an increase in the respective compound. However, level 1 of 4-ethylcatechol was significantly different from all the other levels, which were not significantly different to one another. Conversely, level 5 of isovaleric acid was significantly different from the other levels, which were not significantly different from one another. From this it can be deduced that 4-ethylcatechol suppresses the medicinal descriptor but only when it is present above detection threshold and that isovaleric acid suppresses the medicinal descriptor only at level 5. These suppressant effects account for the slightly lower values found for 4-ethylguaiacol during combination profiling than during singular profiling.

4.1.5 Smoky/Savoury

The results of the ANOVA for the smoky/savoury characteristic are shown in Table 5.21. In terms of the smoky/savoury characteristic, the only significant effect was 4-ethylguaiacol (p = 0.0002). This is slightly unexpected, as 4-ethylcatechol also has a smoky character associated with it and therefore some interaction between the two compounds was expected. The overall effect and means grouping of 4-ethylguaiacol are shown in Table 5.22 and Figure 5.14.

Table 5.21. ANOVA for sensory data obtained regarding the smoky/savoury attribute.

Source	DF	Mean Square	Pr > F
4-EP	4	20.2	0.73
4-EG	3	265	0.0002
4-EC	3	31.3	0.51
ISOV	3	64.3	0.19
4-EP*4-EG	1	2.00	0.82
4-EP*4-EC	1	2.33	0.81
4-EG*4-EC	1	0.347	0.93
4-EP*ISOV	1	4.50	0.74
4-EG*ISOV	1	14.2	0.55
4-EC*ISOV	1	36.1	0.35
4-EP*4-EG*4-EC	1	0.22	0.94
4-EP*4-EG*ISOV	1	23.3	0.44
4-EP*4-EC*ISOV	1	12.5	0.58
4-EG*4-EC*ISOV	1	80.2	0.16
4-EP*4-EG*4-EC*ISOV	1	11.7	0.59
Error	495	40.6	

Table 5.22. Effect of 4-ethylguaiacol on smoky/savoury character of wines.

Level	Means
1	6.2 [b]
2	6.2 [b]
3	6.8 [b]
4	8.1 [b]
5	12.2 [a]
Least Significant Difference (p = 0.05)	**2.85**

[1] Values with the same superscript are not significantly different

A general trend of an increase of smoky/savoury character was observed with an increase of 4-ethylguaiacol. However, the only a significant difference was between the first four levels and level 5. The overall maximum mean was significantly lower than the mean obtained for smoky character when 4-ethylguaiacol was profiled on its own, and this could probably be ascribed to a similar reason as the lower levels obtained for the sick-sweet characteristic during combined profiling.

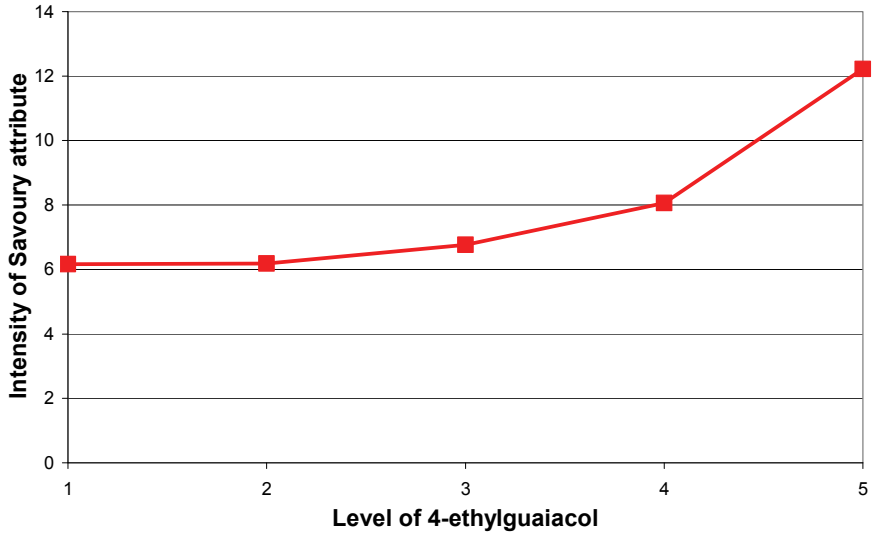


Figure 5.14. Effect of 4-ethylguaiacol on smoky/savoury character in combination with other compounds.

4.1.6 Pungent

The ANOVA for the pungent characteristic is shown in Table 5.23.

Table 5.23. ANOVA on sensory data obtained regarding the pungent attribute.

Source	DF	Mean Square	Pr>F
4-EP	4	266	0.0005
4-EG	3	121	0.078
4-EC	3	47.8	0.43
ISOV	3	609	0.0001
4-EP*4-EG	1	48.3	0.34
4-EP*4-EC	1	11.7	0.64
4-EG*4-EC	1	39.0	0.39
4-EP*ISOV	1	86.7	0.20
4-EG*ISOV	1	396	0.006
4-EC*ISOV	1	45.1	0.36
4-EP*4-EG*4-EC	1	12.5	0.63
4-EP*4-EG*ISOV	1	88.9	0.20
4-EP*4-EC*ISOV	1	6.72	0.72
4-EG*4-EC*ISOV	1	18.0	0.56
4-EP*4-EG*4-EC*ISOV	1	17.0	0.57
Error	495	52.9	

It was found that there was a significant interaction between isovaleric acid and 4-ethylguaiacol ($p = 0.0064$). This interaction is shown in Figure 5.15 ($z = 93.03 + 8.79 x + 192.3$

lny - 2.87 x^2 -142.0$(lny)^2$ -6.88xlny + 0.00600x^3 +35.67$(lny)^3$ + 58.14 x$(lny)^2$ + 1.43x^2lny R^2 = 0.73). It can be seen that the pungent characteristic generally increased with an increase in isovaleric acid concentration. However, there was an interaction where isovaleric acid was at low levels and 4-ethylguaiacol above its detection threshold. 4-ethylguaiacol enhanced the pungency effect of below-threshold levels of isovaleric acid when it is around or slightly higher than detection threshold.

As can also be seen in Table 5.23, 4-ethylphenol also displayed a significant main effect (p = 0.0005). Figure 5.16 and Table 5.24 show that level 4 (1711 µg/L) differed significantly from levels 1 (82 µg/L) and 5 (4695 µg/L), but none of the other levels differed significantly. This may be because 4-ethylphenol has some synergistic effect with isovaleric acid.

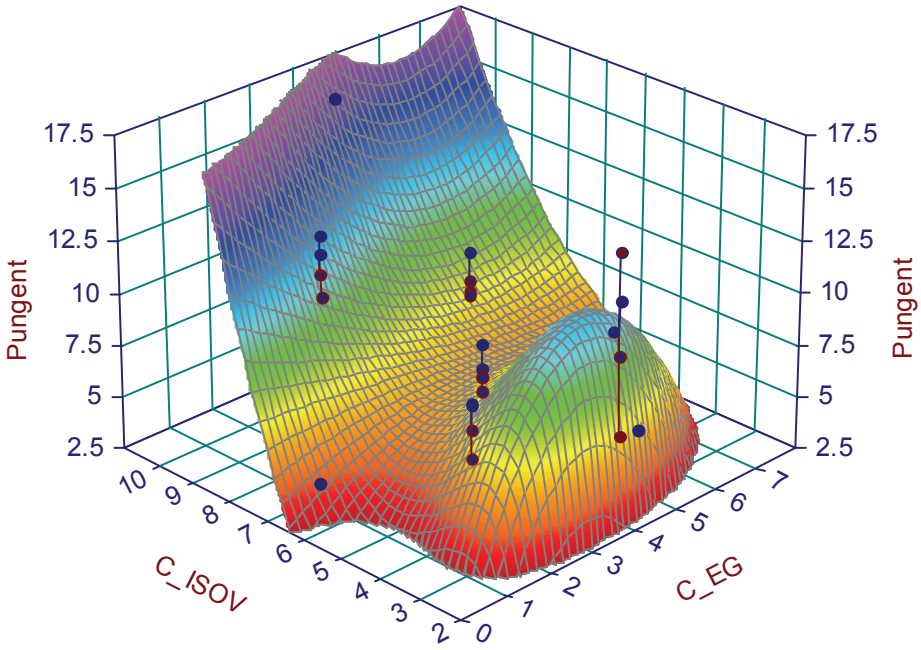


Figure 5.15. Interaction between 4-ethylguaiacol and isovaleric acid for pungent attribute. C_ISOV designates the design level of isovaleric acid, and C_EG designates the design level of 4-ethylguaiacol.

Table 5.24. The effect of 4-ethylphenol on pungent character of Pinotage red wine.

Level	Means
1	4.8 [b]
2	6.9 [a b]
3	6.7 [a b]
4	9.6 [a]
5	5.6 [b]
Least Significant Difference (p = 0.05)	**3.25**

[1] Values with the same superscript are not significantly different

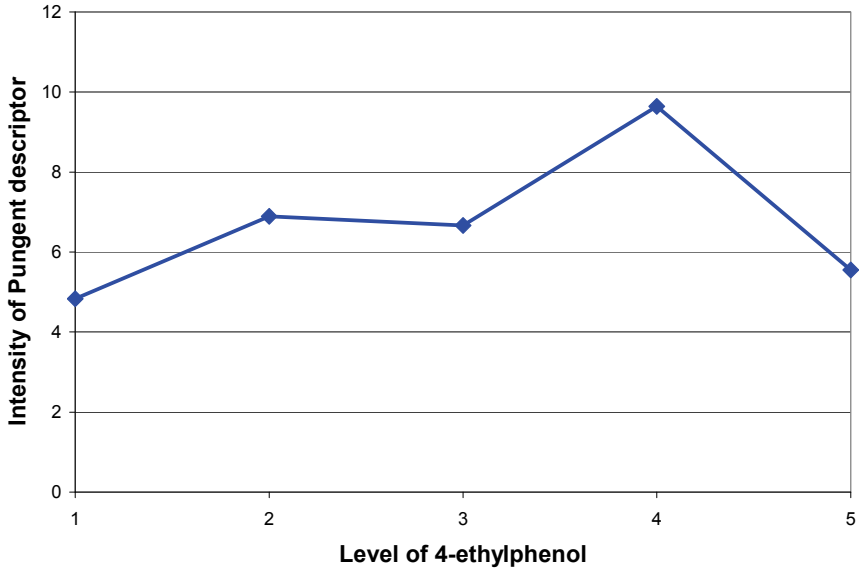


Figure 5.16. Effect of 4-ethylphenol on the pungent characteristic.

4.1.7 Overall effects using different methods of multivariate analysis

The data obtained were investigated using PCA, PARAFAC and PLS. The results obtained from these different analysis methods are presented in this section. Please note that in this section reference is made to quadrants. The first quadrant is (+;+), the second (-;+), the third (-;-) and the fourth (+;-).

Principal Component Analysis (PCA)

A Principal Component Analysis (PCA) biplot of the overall effects of the different compounds is shown in Figure 5.17. Factor (F) 1 explained approximately 38 % of the total variance, and F2 explained approximately 29.5 % of the total variance, with approximately 67.5% of the total variance explained by these two components. It can be seen that the descriptors medicinal and savoury associate with one another (quadrant 2) and sick-sweet and Elastoplast™ associate with one another (quadrant 1). The pungent descriptor associates strongly with F1, and the berry-like descriptor dominates quadrant 3. These associations already make sense, as 4-ethylguaiacol was found to be the strongest causal agent for both the sick-sweet and savoury characters. The association of sick-sweet with Elastoplast™ may have been due to the interaction between 4-ethylphenol and 4-ethylcatechol that was present for both these characteristics. However, this cannot be conclusively stated. The position of the berry-like descriptor is also as expected, as the presence of each of the individual compounds have been associated throughout the study with a decrease in berry-like character, and it is therefore expected that berry-like should not associate with any of the other descriptors. In fact, it is to

some extent negatively associated with Elastoplast™ and sick-sweet, and to a lesser extent to medicinal and savoury.

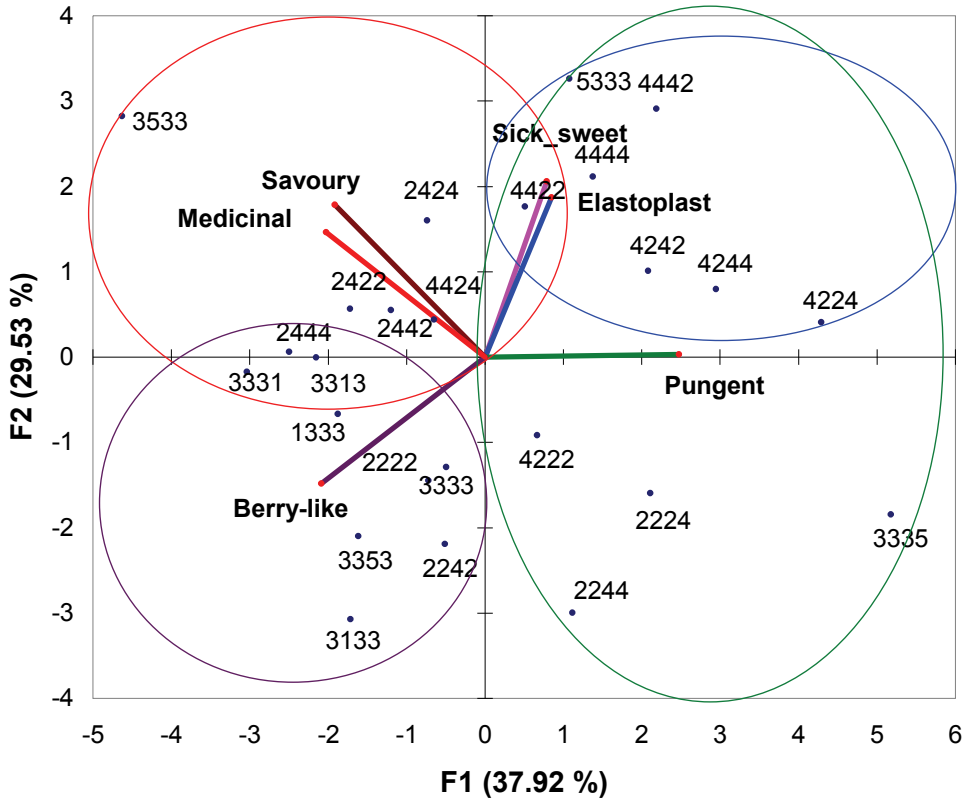


Figure 5.17. PCA Biplot showing 25 samples and all descriptors (berry-like, sick-sweet Elastoplast™, medicinal, savoury, and pungent) used during combination profiling. Groups of samples scoring highest in specific descriptors are indicated. 67.5% of the variance is explained by the two components.

Figure 5.17 also shows several sample groupings. The groups that were identified as significantly different from the other samples for each descriptor were identified, and circled on Figure 5.17. It is interesting to note that the specific descriptors divide the PCA biplot into approximately four quadrants. Elastoplast™ spans the first quadrant, medicinal spans the second quadrant (with the inclusion of three samples that lie on the border of this quadrant), berry-like spans the third quadrant (with the inclusion of the same three "border" samples) and the pungent descriptor spans the first and fourth quadrants. The slight overlap of the medicinal and berry-like characters is an interesting occurrence, as 4-ethylguaiacol was not found to be a significant contributor to berry-like character, and a negative association would be expected. However, all three the other compounds caused a decrease in the berry-like descriptor (4-ethylphenol and 4-ethylcatechol were involved in a significant interaction, and isovaleric acid was a statistical main effect, see Table 5.5, Section 4.1.1), explaining the positive association. The position of pungent in relation to the other descriptors can be explained as follows: it is in

the opposite direction to the medicinal and savoury characteristics, but in the same half as the Elastoplast™, berry-like and sick-sweet characteristics. This may be because 4-ethylguaiacol, the causal agent for the medicinal characteristic, did not contribute to the pungent characteristic. The weak association that loading for the pungent attribute shows with that of the Elastoplast™ attribute may be explained by the fact that 4-ethylphenol, a compound associated with the Elastoplast™ attribute in Chapter 4, was identified as a statistical main effect in terms of the pungent descriptor.

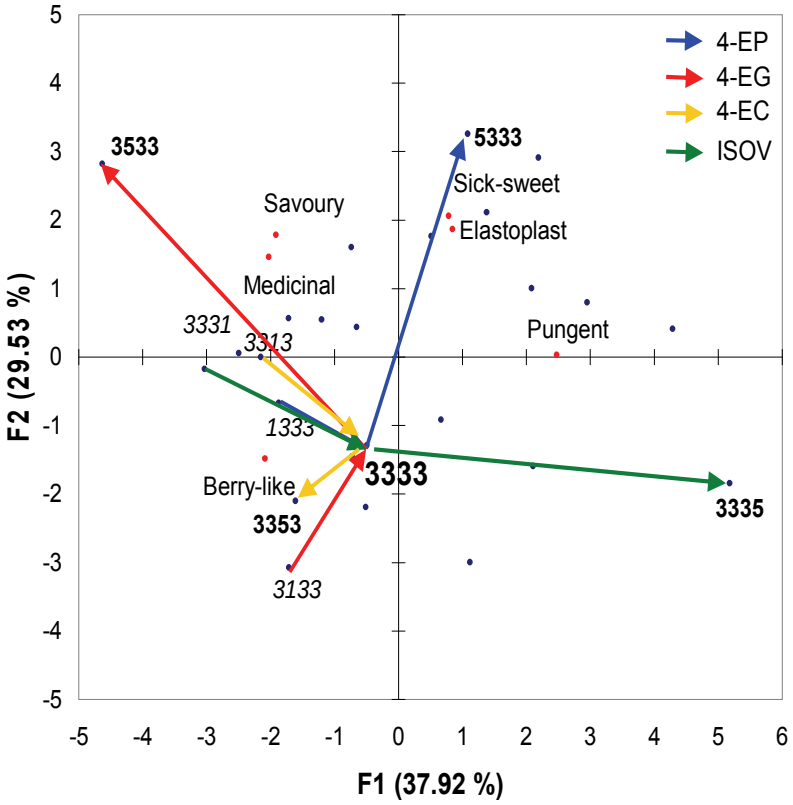


Figure 5.18. PCA biplot of all descriptors (berry-like, sick-sweet, Elastoplast™, medicinal, savoury, and pungent) and some star and centre samples used during combination profiling showing change in aroma profile of samples with change from lowest to highest levels of each compound (4-ethylphenol, 4-ethylguaiacol, 4-ethylcatechol and isovaleric acid). 67.5% of the variance is explained by the two components.

Figure 5.18 shows the change in aroma profile with the change from lowest concentration to highest concentration of the compounds tested. Only the star and centre samples are annotated. The samples containing the lowest concentrations are shown in italics, whereas the samples containing the highest concentrations are shown in bold. It can be seen that for 4-ethylphenol, 4-ethylguaiacol and isovaleric acid, the samples move in the directions of the relevant descriptors when the concentrations were increased from the centre sample (level 3) to the sample containing level 5 of each compound. In the cases of 4-ethylphenol and 4-ethylguaiacol, these samples move in the same direction of samples which have their

concentrations increased from level 2 to 4 (not shown). It is interesting to note that 4-ethylcatechol moves in the direction of the berry-like descriptor. However, the reason for this is not apparent.

A very interesting aspect of Figure 5.18 is the angles between the vectors – in other words the differences in direction of change between samples changing from below what is considered detection threshold to above detection threshold (from level 1 to level 3) and from above detection threshold to an extreme level (level 3 to level 5). If no interactions were present, one would expect movement of the samples to be in the similar direction. However, this is only the case for isovaleric acid. It is likely that this was due to the base concentration of isovaleric acid found in all red wines. In the cases of 4-ethylphenol, 4-ethylguaiacol and 4-ethylcatechol, the movement of the samples from level 1 to level 3 is perpendicular to the movement from level 3 to level 5. In the cases of 4-ethylphenol and 4-ethylguaiacol – where the samples move in the direction of the relevant descriptors with an increase from level 3 to level 5 – it implies that an increase in concentration from below detection threshold to above detection threshold affects the aroma profile, but not in terms of the relevant descriptors.

Another noteworthy aspect of Figure 5.18 is the difference in size of these vectors. For 4-ethylphenol, 4-ethylguaiacol and isovaleric acid, the change from level 1 to level 3 is much smaller than the change from level 3 to level 5. This implies that the effect on the aroma profile is greater when the concentrations of these compounds are at their extreme levels. The latter is expected. What is somewhat unexpected, however, is the fact that the change of 4-ethylcatechol from level 3 to level 5 is smaller than the change from level 1 to level 3. This means that the presence of 4-ethylcatechol at levels higher than threshold has a large sensory effect on the overall profile of a wine, but increasing the concentration of 4-ethylcatechol above this level does not have an additional effect. It is also interesting to note the strong association of the sample containing the highest level of 4-ethylcatechol (3353) and the berry-like descriptor.

A final remarkable aspect of Figure 5.18 is the similarity of the direction of movement from levels 1 to 3 for 4-ethylphenol, 4-ethylcatechol and isovaleric acid. 4-ethylguaiacol moves in a direction that is almost perpendicular to these three compounds and in the opposite direction to the berry-like descriptor. It is tempting to conclude that 4-ethylguaiacol had the greatest suppressant effect on berry-like character. Yet, as previously discussed, 4-ethylguaiacol is neither a main effect for this descriptor, nor part-takes in a significant interaction for this attribute. The explanation to this observation, however, lies with the medicinal descriptor. The movement of 4-ethylphenol, 4-ethylcatechol and isovaleric acid from level 1 to level 3 are in exactly the opposite direction to the medicinal descriptor. The suppressant effects of these compounds on the medicinal aroma of 4-ethylguaiacol (as described in section 4.1.4) therefore explain these different directions.

PARAFAC

PARAFAC was performed on the raw dataset that contained the responses of all the judges. This method was applied as it allows for simple multivariate exploration of the data, and produces an easily interpretable model from all the sensory responses. The main advantage of this is that the inherent variability between the judges is taken into account by this model, as it can be performed on the raw sensory data (Bro *et al*., 2008).

It was found that three factors produced a model with a core consistency of 95%. Although a two-factor model gave a higher core consistency (100%), relevant sensory information could still be extracted from the third factor, and thus the three-factor model was chosen. This three-factor model explained 46.6% of the variation in the dataset. Note that this value is lower than the 67% that could be explained by PCA, but due to the difference between PCA and PARAFAC, it is likely that the excess variation explained by PCA is noise. Figure 5.19 shows the loading plot for factors 1 and 2 of the sensory variables, whereas Figure 5.21 shows the loading plot for factors 2 and 3 for the same set of variables.

From Figure 5.19 two variables are identified as having a large effect on the variation of the dataset, namely Elastoplast™ (factor 1) and berry-like (factor 2). Note that the rest of the variables are clustered together, indicating that these variables do not have such a large effect on the overall variation of the dataset.

The importance of Elastoplast™ can be explained by the fact that this descriptor could easily be identified by all the panel members, and that its character is distinctly different from all the other descriptors in this study. It is also interesting to note that this characteristic is commonly linked to Brett character in literature (Chatonnet *et al*., 1992; Wirz *et al*., 2004; Romano *et al*., 2009). As can be seen in the scores plot (Figure 5.20), the sample most strongly associated with the Elastoplast™ descriptor (5333) contained both the highest level of 4-ethylphenol, and had the highest intensity of the Elastoplast™ attribute. The other sample that also appears to be "driving" this factor is 4244, which had the second highest mean for the Elastoplast™ attribute.

The berry-like attribute is one that is suppressed by the presence of all the respective compounds used during this study. This is not only apparent from this study, but also the one described in Chapter 4. A decrease in fruitiness is also one of the sensory effects commonly associated with Brett character (Licker *et al*., 1999; Fugelsang & Zoecklien, 2003; Ugarte *et al*., 2005; Fariña *et al*., 2007; Cliff & King, 2009). As all the samples contained all four the compounds, a degree of suppression in berry-like character is expected in all the samples. It is therefore logical that this attribute drives factor 2, as this descriptor is relevant in all of the samples used in this study. Figure 5.20 shows a group of samples driving the variation in the direction of the berry-like attribute. These samples are samples 3133, 2244, 2242, and 1333. This sample grouping is notable for two reasons. Firstly, although this group contains the

sample with the highest mean in terms of berry-like character (3133), this group does not directly correspond to the four samples with the highest level of berry-like character (they are numbers 1, 4, 6 and 8 in the ranking). The PCA plot, (Figure 5.17) also does not show a similar grouping to Figure 5.20. This indicates that the methodology used for data analysis prior to PCA (i.e. finding the means of all the judges) confounded important information about this grouping.

Figure 5.21 shows the sensory loadings plot for factor 2 versus factor 3, and the corresponding scores plot is shown in Figure 5.22. It can be seen that factor 3 is driven by two attributes, namely medicinal and pungent. This is logical, as each of these descriptors is linked to a specific compound used in the study, and drive PC 2 in Figure 5.17. This means that although these two attributes are not the main causes of variation in the dataset, some of the overall variation can be attributed to them.

When referring to the scores plot (Figure 5.22), two interesting aspects can be observed. Firstly, the sample containing the highest level of 4-ethylguaiacol (3533) has a high score in the latent variable described by the medicinal descriptor. This is logical, as this compound is linked to the medicinal attribute, and had the highest mean for this attribute. This separation can also be seen in the PCA plot (Figure 5.17). There is also a general trend of samples with high levels of 4-ethylphenol (i.e. sample codes starting with either a 4 or a 5) to fall in quadrant 3. However, no such pattern could be observed with samples containing high levels of isovaleric acid. This indicates that these samples tend to associate with the pungent descriptor. This is interesting, as a general increase in the pungent attribute could be seen with an increase in the level of 4-ethylphenol (see Figure 5.16). From this, it can be concluded that 4-ethylphenol enhances the pungency associated with isovaleric acid. This sensory interaction may be the underlying cause for the conflicting results found in studies regarding isovaleric acid and Brett character (Fugelsang & Zoecklein, 2003; Romano *et al*., 2009).

When interpreting Figure 5.19 and Figure 5.21, it can be concluded that the sick-sweet descriptor was not important in the dataset, as none of the three factors identified by the PARAFAC model showed variation due to this descriptor. As described in Chapter 4, this attribute mainly comes about from the suppression of the natural berry-like character of the wine, but may not be as perceived intensely when several other sensory factors are present. This corresponds to literature, as the sick-sweet attribute has not yet been coupled to Brett character. In Figure 5.17, the sick-sweet attribute correlates strongly with the Elastoplast™ descriptor, and this figure can easily be interpreted incorrectly by interpreting the trends exhibited for the Elastoplast™ descriptor as if they applied to the sick-sweet attribute. In this study, this was prevented by an extensive knowledge of the samples in the study and the literature involved. However, the results of PARAFAC pose none such dangers of incorrect interpretation.

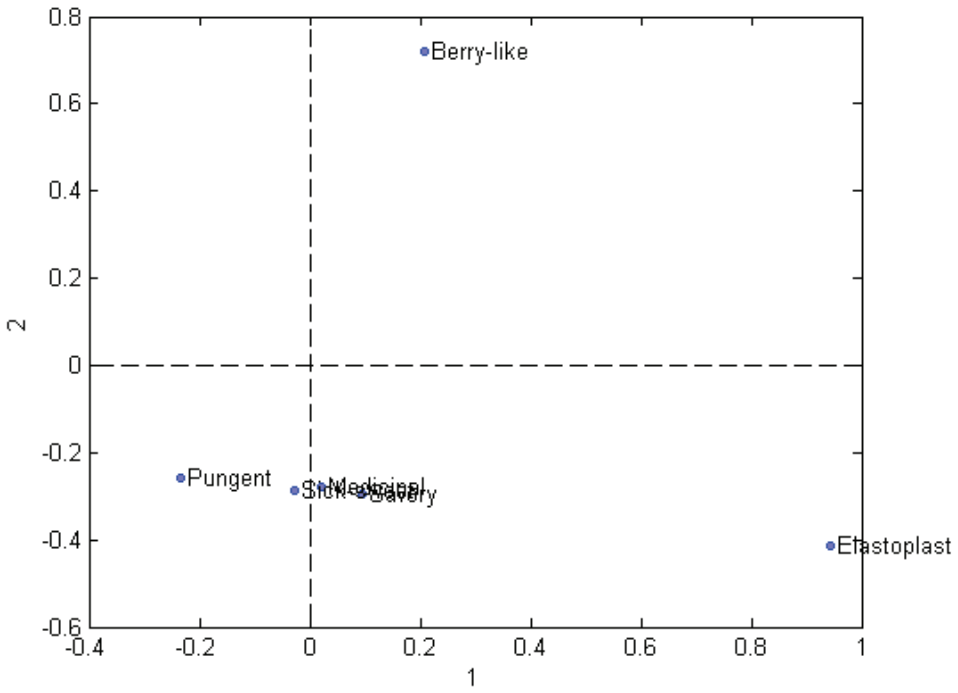


Figure 5.19. Plot of factor 1 versus factor 2 for sensory loadings in PARAFAC performed on raw sensory data obtained from profiling samples containing varying levels of Brett-related compounds. Note that the loadings for the sick-sweet, medicinal and savoury attributes associate with one another.

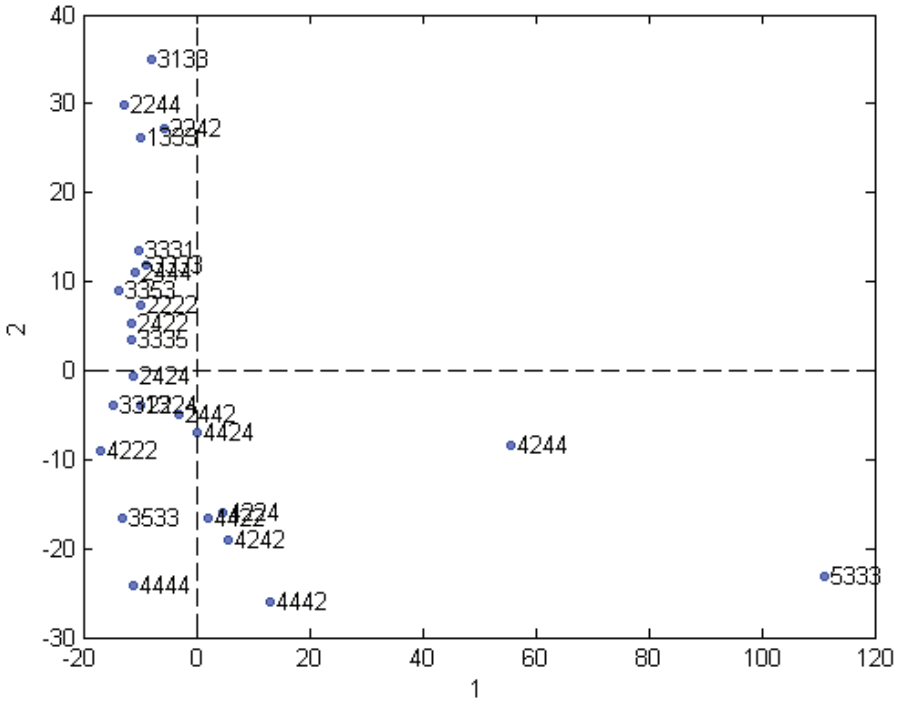


Figure 5.20. Scores plot for factor 1 versus factor 2 in the PARAFAC model obtained of raw data of sensory profiling of samples containing different levels of Brett-related compounds. Note that the scores are labelled as described in Section 3.2.

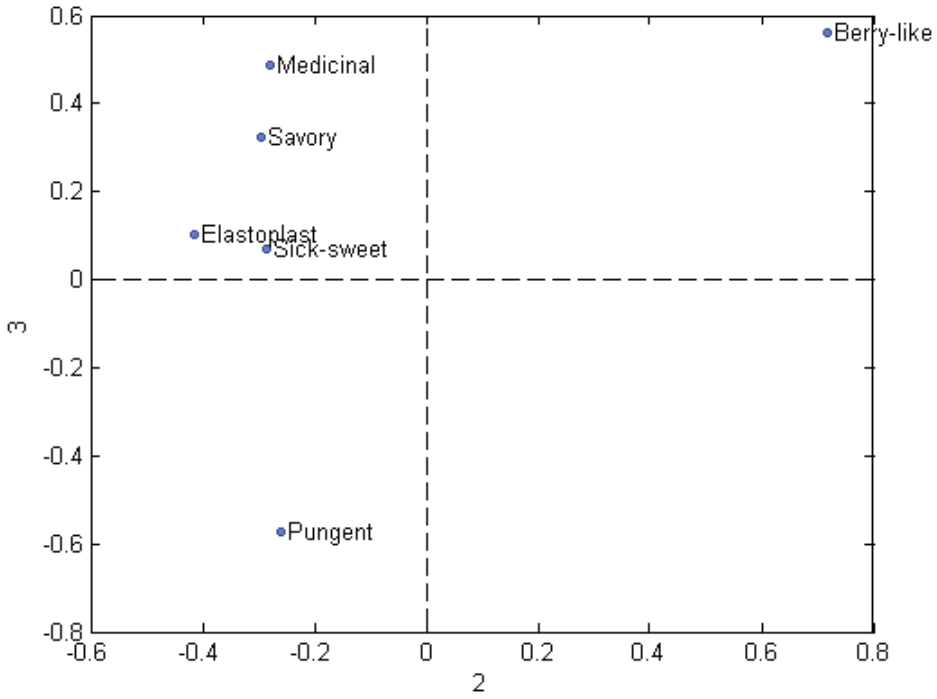


Figure 5.21. Plot of factor 2 versus factor 3 for sensory loadings in PARAFAC performed on raw sensory data obtained from sensory profiling of all combinations of Brett spoilage compounds used in this study.

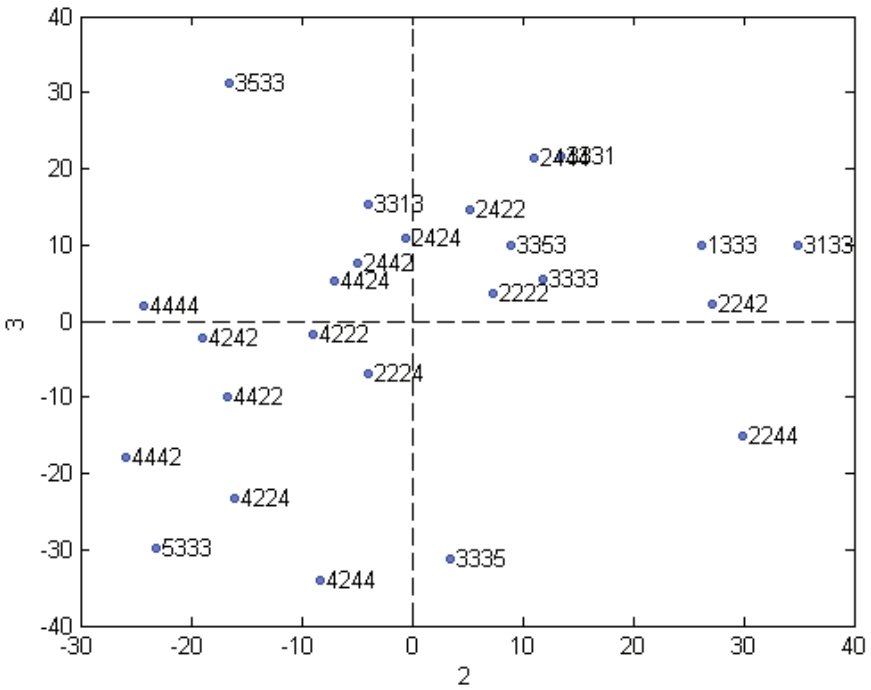


Figure 5.22. Scores plot of factor 2 versus factor 3 in the PARAFAC model obtained from raw sensory data of samples containing different levels of Brett-related compounds. Note that the scores are labelled as described in Section 3.2.

The results found in the PARAFAC appear to give different results than PCA, but the overall conclusions remain the same. The results from PARAFAC are, however, clearer than those obtained from PCA, and provide a stronger hierarchy in terms of sensory variables. PARAFAC was complementary to PCA as it allowed for better interpretation of the overall dataset. PARAFAC can also aid in preventing incorrect conclusions to be drawn from PCA. This makes sense as the PARAFAC model is more focussed on modelling the systemic variation and less likely to model noise (Bro, 1997).

Partial Least Squares Regression (PLS)

A Partial Least Squares (PLS-2) analysis was also performed using XLStat (Addinsoft, 2009). This analysis was not performed to attempt to set up a predictive model, but rather to explore the relationships between the different levels of (placed in the X-space) and the sensory descriptors (placed in the Y-space). This approach was taken, since the different levels of the compounds should cause different sensory qualities.

As the compounds had been shown to have diverse effects at different levels, the chemical composition was set as a category variable. Five components were modelled. Component 1 and 2 modelled 42% of the variance, whereas component 2 and 3 modelled 43% of the variance and component 1 and 3 modelled 37% of the variance. Plots of component 1 versus component 2 and component 1 versus component 3 showed a clear separation of scores and X-loadings according to whether they were “cube” or “star” samples and could therefore be related to the design (not shown), and therefore provided no additional information. It can thus be inferred that component 1 models the design and not the data, and that more information could be obtained from modelling component 2 versus component 3. This can be seen in Figure 5.23. Please note that Figure 5.23 is flipped along both PC 2 and PC 3 in order to allow comparison with PCA. The rotation in PCA is of an arbitrary nature.

An interesting aspect of Figure 5.23 is the fact that the Y-loadings follow a similar pattern to the loadings when a PCA was performed (Figure 5.17). Several patterns amongst the X-loadings were also observed. The X-loadings for the higher levels (levels 4 and 5) of 4-ethylphenol and 4-ethylguaiacol correlated with the descriptors relevant for those compounds (Elastoplast™ for 4-ethylphenol, and medicinal and savoury for 4-ethylguaiacol). Level 5 of isovaleric acid also correlated to the pungent descriptor. These relationships are similar to those observed in Chapter 4. The X-loadings corresponding to the lowest level (level 1) of all of these compounds tend to fall in the centre of the biplot, which means that these levels did not have a large effect on the overall sensory profile. This is not unexpected, as these compounds are all below detection threshold and are therefore not expected to make much of a difference to the overall sensory profile. The loadings for 4-ethylcatechol also tend to fall towards the centre of

the plot, which means that the 4-ethylcatechol did not have a large overall effect on the sensory profiles of these wines.

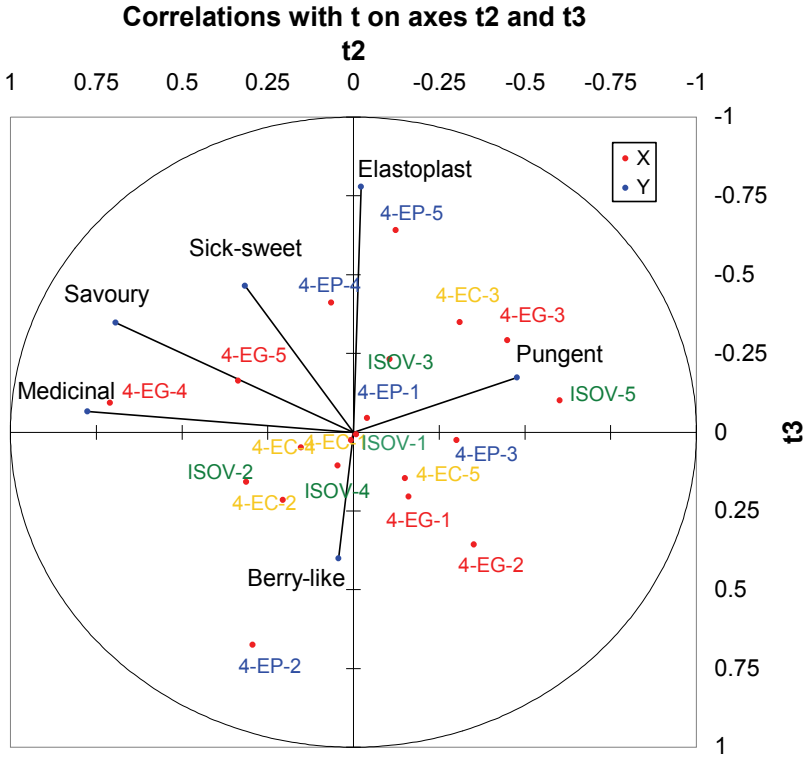


Figure 5.23. Plot of X (chemical) and Y (sensory) loadings in components 2 and 3 obtained by PLS. Note that the scales in this figure appear in the opposite direction. The level of each compound is designated by the last character in the X-loading label. For example, 4-EP-3 designates level 3 of 4-ethylphenol.

However, some of the X-loadings do not fall in the expected patterns. The first of these are 4-ethylguaiacol at levels 2 and 3, which correlate negatively with the medicinal descriptor and appear to form a positive correlation with the pungent descriptor. This may be due to the enhancement effect the 4-ethylguaiacol was found to have on pungency when present at lower levels (see section 4.1.6). The position of the loading that seems the most arbitrary is that of 4-ethylphenol level 2, which correlates negatively with the Elastoplast™ descriptor and positively with the berry-like descriptor. However, this can be explained when the scores of this PLS is investigated (Figure 5.24).

As can be seen in Figure 5.24, the scores group together according to the design. The cube samples form four distinct groups that are divided according to their 4-ethylphenol and 4-ethylguaiacol concentrations. Interestingly, within these groups, a pattern according to the concentrations of 4-ethylcatechol and isovaleric acid can also be observed. The position of level 2 of 4-ethylphenol on Figure 5.23 and its association with berry-like character is therefore not because level 2 of 4-ethylphenol causes berry-like character, but due to the berry-like descriptor

associating strongly with the samples containing level 2 of both 4-ethylphenol and 4-ethylguaiacol.

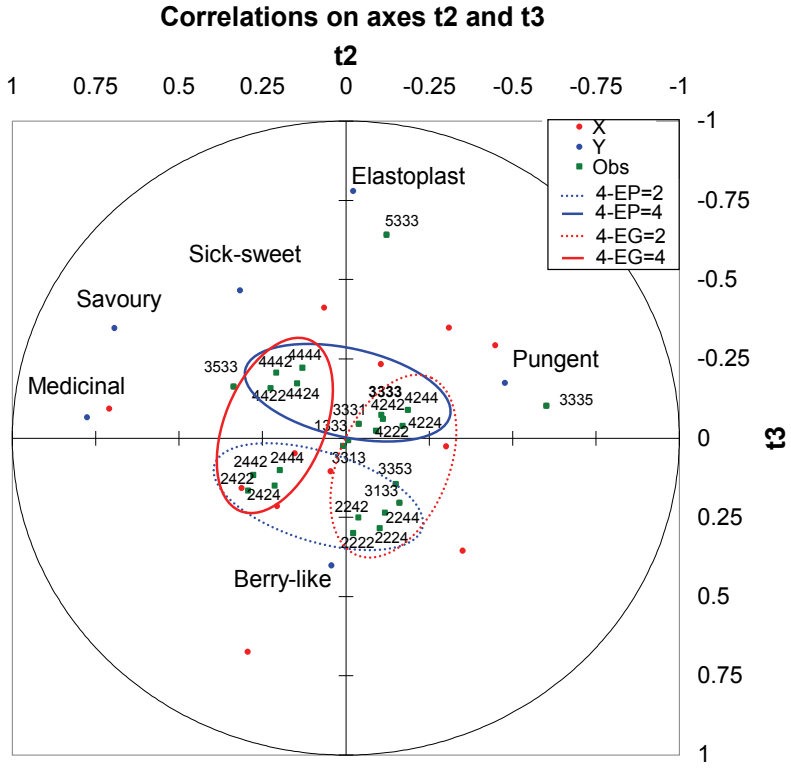


Figure 5.24. PLS plot of t2 and t3 containing X-loadings, Y-loadings (sensory descriptors; berry-like, sick-sweet, Elastoplast™, medicinal, savoury, and pungent) and scores (25 samples). Grouping according to 4-ethylphenol and 4-ethylguaiacol content is indicated.

4.2 Consumer analysis

Of the 100 consumers surveyed, 75 % fell in the age group of between 18 and 25. Sixty-nine percent of the consumers consumed wine at least once a week, and 91% of the consumers drank wine at least twice per month. Approximately half of the consumers indicated that they most often consumed Pinotage. In terms of wine knowledge, 48 consumers considered themselves novice drinkers, whereas 51 consumers felt they had a fair amount of wine knowledge. None of the consumers that participated in this study considered themselves wine experts.

For the total group of consumers, the sample effect was significant ($p = 0.0050$), which means that the variation in the dataset could be ascribed to differences between the samples and not just to the inherent differences between consumers. When the analysis was repeated on the novice consumers (group A), it was found that sample was not a significant effect. However, the sample effect was highly significant ($p = 0.0051$) with the remaining group of consumers (group B). From this, it can be deduced that the effect of sample in the total group

was caused by this group of consumers. The difference in effect can also be ascribed to the fact that novice wine drinkers respond differently to wine and wine faults than more experienced wine drinkers (Prescott *et al*., 2005).

External preference mapping was performed, and can be seen in Figure 5.25. This method first maps the descriptive data in the X-space (using PCA), and then subsequently plots the preference data onto this map obtained (Tenehaus *et al*., 2005; Meilgaard *et al*., 2007). The samples with the highest and lowest levels of the different compounds (star samples) are shown in bold. If a sample contained level 4 of a compound, it is presented in colour. Blue indicates 4-ethylphenol; red indicates 4-ethylguaiacol, yellow 4-ethylcatechol and green isovaleric acid. As can be seen, the samples that contain level 4 of 4-ethylphenol group together, and the samples containing level 4 of 4-ethylguaiacol group together. Both these groups fall way from the direction of liking, and from this it can be interpreted that both these compounds have a negative effect on liking. It is also interesting to note that liking falls in the opposite direction to the medicinal descriptor, which is associated with high levels of 4-ethylguaiacol in wine (Chapter 4).

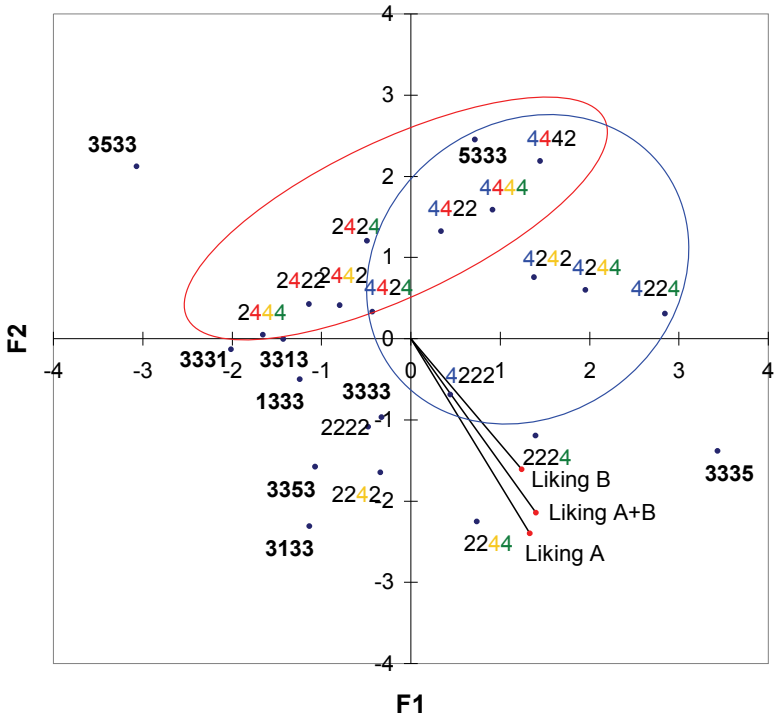


Figure 5.25. Preference map 25 of samples obtained during consumer analysis using 100 consumers. A refers to the "inexperienced" consumer group, whereas B refers to the "experienced" consumer group. Samples containing level 4 of each compound (4-ethylphenol, 4-ethylguaiacol, 4-ethylcatechol and isovaleric acid) are shown. Grouping according to 4-ethylphenol and 4-ethylguaiacol content is indicated. 67.5% of the variance is explained by the two components.

It was expected that the liking of the consumers would fall in a similar direction than the berry-like descriptor, as the spoilage compounds all suppress berry-like character to some degree and wine consumers consider the absence of fault as an important quality characteristic in wine (Charters & Pettigrew, 2007). This, however, was not the case, as liking fell in a direction that was not associated with any of the descriptors used in this study (Figure 5.26), which implies that there is a secondary or tertiary characteristic driving liking in this sample set.

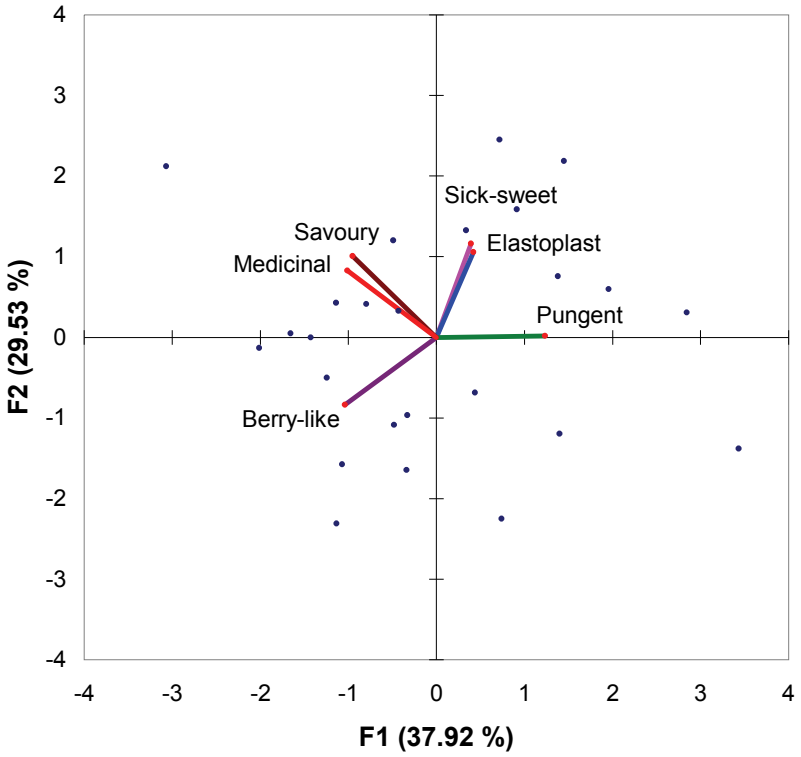


Figure 5.26. Scores and loading plot for preference map obtained from the data obtained from 100 consumers showing sensory loadings (berry-like, sick-sweet, Elastoplast™, medicinal, savoury and pungent). 67.5% of the variance is explained by the two components.

PLS based preference mapping was also performed on the sensory and consumer data and the map can be seen in Figure 5.27 and Figure 5.28. In this method, the sensory descriptive data is in the X-space, and the consumer data is in the Y-space. The principal components were therefore determined from the variation present in both these data sets and not just the variation in the descriptive data, as is the case with external preference mapping.

When comparing the liking (Y) loadings and the descriptive (X) loadings in Figure 5.27, two interesting aspects were revealed. Firstly, the liking vector for the “experienced” group of consumers (Group B) lie in exactly the opposite direction of the Elastoplast™ descriptor, which is commonly associated with Brett character. It can therefore be postulated that consumers with a higher level of wine knowledge find this characteristic most objectionable. Secondly, the liking vector for the “inexperienced” group of consumers (Group A) lie in the opposite direction to the medicinal characteristic, indicating that consumers with a lower degree of wine knowledge find

the uncharacteristic medicinal aroma more unpleasant than other wine consumers. These findings are in line with those of Curtin *et al*. (2008), who found strong negative correlations between “medicinal” and “leather” aromas in wine and consumer liking

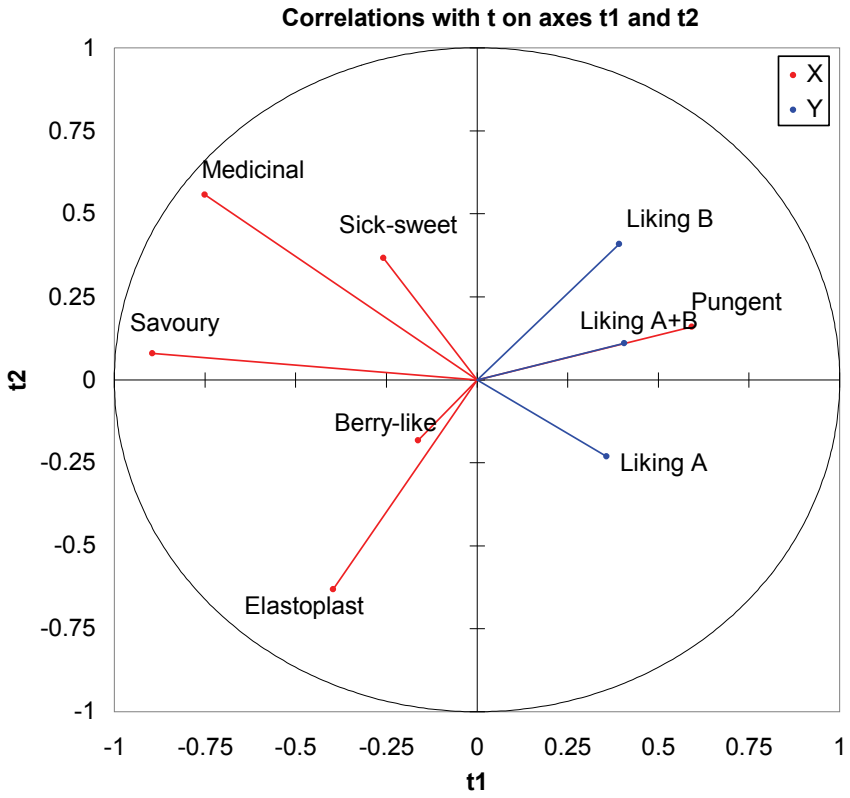


Figure 5.27. PLS-based preference map for all samples showing sensory descriptors (berry-like, sick-sweet, Elastoplast™, medicinal, savoury and pungent) and inexperienced (A) and experienced (B) consumer groups.

Figure 5.28 shows both the scores and the loadings for the PLS done on the consumer data. The overall liking for the experienced group of consumers fall in an area that contains no samples, which indicates that none of the samples were liked by the experienced consumers. It can thus be concluded that Brett tainted wines are more disliked by consumers with a level of wine expertise than casual wine drinkers.

In Figure 5.28, the scores are divided according to their 4-ethylguaiacol content. It can be seen that the scores for a level 4-ethylguaiacol level of higher than 4 tend to group together. Star samples in this group all contained the lowest level of the different compounds. No such grouping could be observed in terms of any of the other compounds. It could therefore be inferred that the level of 4-ethylguaiacol is a driver of the liking of the inexperienced consumer group.

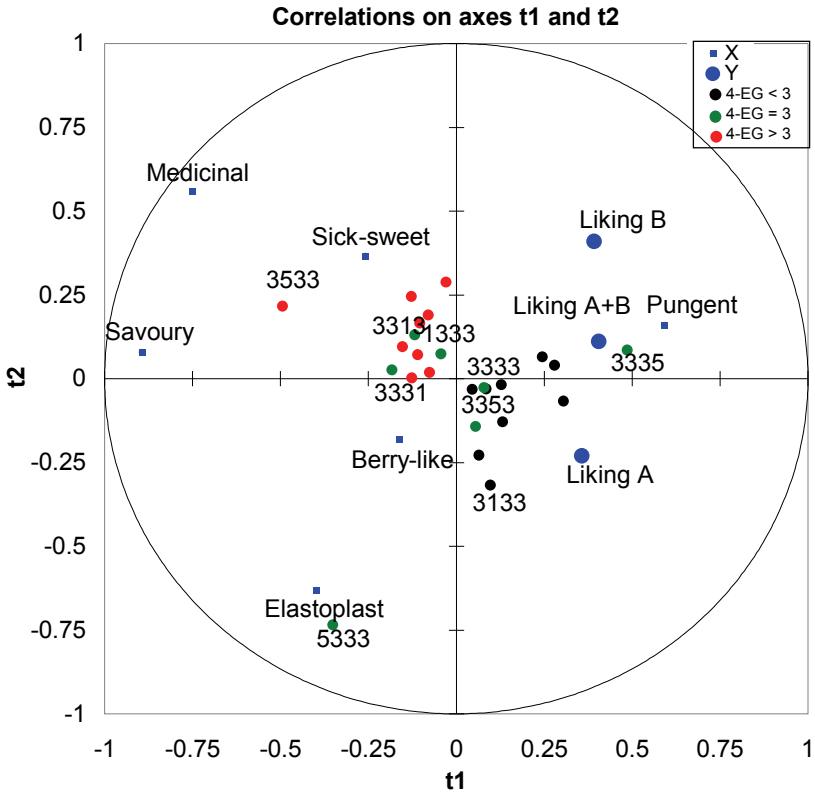


Figure 5.28. PLS scores and loadings plot for PLS showing all descriptors (berry-like, sick-sweet, Elastoplast™, medicinal, savoury and pungent), 25 samples and inexperienced (A) and experienced (B) consumer groups. In this figure, only the loadings for the centre and star samples are labelled.

It is notable that although such a small amount of variance could be modelled by PLS, the liking patterns of consumers could be explained a lot better than through external (PCA-based) preference mapping. This is because PCA in the external preference map is based solely on the sensory attributes of the samples, and disregards the fact that consumer liking may not be coupled to a specific sensory attribute, but rather to the manner in which the sensory attributes relate to one another. Although the number of samples used in this study made it impossible, is likely that more of the variance in the dataset could be explained if all consumers were to receive all the samples and could then be mapped individually or via cluster analysis.

When the liking scores for the different samples of wines are explored in more detail, some interesting aspects are revealed. The mean liking scores for consumer group B are shown in Table 5.25.

In Table 5.25 it can be seen that the two samples that were least liked are the two containing the lowest level of 4-ethylphenol (1333) and the highest level of 4-ethylphenol (5333) respectively. When referring to the positions of these samples on Figure 5.25, it can be seen that these samples correlate neither to each other nor negatively to Liking. The poor correlation between the two samples seems obvious as they respectively have the highest and lowest

intensity of the Elastoplast™ descriptor, which is strongly associated with PC2 in Figure 5.17. This is further indication that a property not analysed during descriptive analysis, and therefore not affecting the positions of samples in Figure 5.17, is the main driver of liking in this sample set.

Table 5.25. Mean liking scores for consumer group B. Values having the same superscript are not significantly different from one another.

Sample	Liking mean
2422	6.22 ^{a}
2244	6.00 ^{ab}
2424	5.89 ^{abc}
2222	5.44 ^{abcd}
3331	5.43 ^{abcd}
4422	5.38 ^{abcd}
3335	5.38 ^{abcd}
4442	5.33 ^{abcd}
2442	5.33 ^{abcd}
0000 (control)	5.25 ^{abcde}
2224	5.25 ^{abcde}
4444	5.18 ^{abcde}
3333	5.10 ^{abcdef}
4244	5.00 ^{abcdef}
4222	4.88 ^{bcdef}
3133	4.73 ^{bcdef}
2242	4.69 ^{bcdef}
3353	4.67 ^{cdef}
4224	4.57 ^{cdef}
4242	4.54 ^{defg}
3313	4.36 ^{defg}
4424	4.30 ^{defg}
3533	4.00 ^{efg}
2444	3.80 ^{fg}
1333	3.80 ^{fg}
5333	3.22 ^{g}
Least Significant Difference (p = 0.05)	**1.318**

With further investigation of Table 5.25, it can be seen that most of the “star” samples fall towards the bottom of the table, and that the two lowest “cube” samples are 2444 and 4424. This led to the hypothesis that a concept like balance could be a driver of liking, as this was also found to be an important quality dimension in wine (Charters & Pettigrew, 2007). However, balance was not quantified during the descriptive analysis. As a possible indication of “balance”, the samples were grouped according to the relationship between the levels of the different compounds. The star samples were considered “unbalanced”, as they contained either a very high or a very low level of only one compound. Furthermore, samples that contained only one

compound at a higher or lower level (such as 2444 or 4424) were considered “unbalanced”. This divided the sample set into 16 “unbalanced” and 10 “balanced” samples.

A simple analysis was performed on the distribution of these types of samples. The percentage of the total each of these sample types occurring in the nine most liked and nine least liked samples was investigated. The choice of nine samples was not arbitrary – the first nine samples are all significantly different from the last four samples, and the last nine from the first three samples. Nine samples also comprise approximately 33% of the data set (result not shown).

It was found that 50% of the “balanced” samples occurred in this top group, and 43% of the unbalanced samples occurred in the bottom group. This indicates that the concept of balance may indeed be a driver for consumer acceptance for Brett-tainted samples.

From all these results, it is recommended that future studies regarding preference mapping of wine taints should not only include quantitative descriptive and hedonic data, but also a third type of conceptual data. Risvik *et al.* (2008) have performed studies analysing the way complex concepts relate to the sensory attributes of several food and fragrance products. A technique of this type could be adapted for use in wine taints, using known quality parameters (Charters & Pettigrew, 2007) as well as negative characteristics. This will allow researchers to obtain information about secondary complex quality characteristics –such as body, complexity and balance – which are not obtained during descriptive analysis and may be essential in explaining consumer liking of wines. Inclusion of concepts such as “uncharacteristic of wine” and “spoiled” will also allow researchers to determine whether a sample is considered “tainted” – which could not be determined using conventional quantitative descriptive analysis.

5 CONCLUSIONS

Previous results (Chapter 4) showed that 4-ethylphenol, 4-ethylguaiacol, 4-ethylcatechol and isovaleric acid suppress berry-like character and cause an increase in respectively the Elastoplast™, medicinal, savoury and pungent descriptors. This is in line with findings in literature. However, when these compounds are combined in wine, they no longer act as expected or predicted from other studies done on their detection thresholds. For example, Chatonnet *et al.* (1992) found that the detection threshold of 4-ethylphenol in red wine was lower when combined with 4-ethylguaiacol than when on its own. For this reason, one would expect an enhancement effect to occur between the sensory effects of these two compounds. However, it was found that 4-ethylguaiacol suppresses the Elastoplast™ attribute associated with 4-ethylphenol, and that 4-ethylphenol has a slight suppressant effect on the medicinal character of 4-ethylguaiacol. Similarly, Romano *et al.* (2009) found that the presence of isovaleric acid increased the detection threshold of 4-ethylphenol, and one would therefore expect the Elastoplast™ descriptor to be suppressed by this compound. Finally, Larcher *et al.*

(2008) postulated that 4-ethylcatechol probably does not have a detrimental effect on the character of wine. Nonetheless, this study found it to have an enhancement effect on the Elastoplast™ character associated with 4-ethylphenol. These findings make sense in the light of the fact that studies done of simple mixtures can generally not be extrapolated to multi-component mixtures (Brossard *et al.*, 2007).

PARAFAC was used as a complementary tool to PCA, and it was found that the panel could find the largest differences in the Elastoplast™ and berry-like attributes. This may be because the Elastoplast™ attribute is severely different from the rest of the attributes profiled, and the fact that the berry-like attribute was relevant in all the samples profiled. PARAFAC also revealed that the sick-sweet attribute was of low importance in the samples. Additionally, PARAFAC identified a group of berry-like samples that could not be identified by either univariate statistics or PCA, and could aid in the explanation of the nature of the effect of 4-ethylphenol on the pungent attribute. It is therefore recommended that PARAFAC be used to complement PCA in future sensory studies.

An attempt was made to map the preference of consumers for wines containing compounds related to Brett character, and three important findings were made. Firstly, some of the tainted samples were found to be more acceptable to consumers than the base wine (Table 5.25). This was expected, as it is generally accepted that Brett character adds complexity to wine (Saurez *et al.*, 2007), which can increase the quality of wine (Charters & Pettigrew, 2007). Secondly, inexperienced wine consumers did not distinguish between the samples in terms of liking, whereas more experienced consumers detected such differences. This is in line with findings of other studies dealing with wine taints (Prescott *et al.*, 2005), and it is recommended that further studies regarding consumers and wine faults such as Brett character should take this into consideration. Thirdly, it was found that the sample containing the highest level of 4-ethylphenol was liked the least by consumers, and it can therefore be said that samples containing a high level of Brett character are objectionable. This is in line with the findings of Lattey *et al.* (2007) and Curtin *et al.* (2008), who also found samples with the highest degree of Brett character least liked by consumers.

However, the consumer analysis failed to accurately map the consumer liking of these tainted samples according to sensory descriptors or concentrations of spoilage compounds. It is speculated that consumer liking of these samples is driven by a secondary sensory characteristic such as wine balance or complexity. It is therefore recommended that future studies of this type include conceptual analysis on wines.

This study further underlines the fact that sensory Brett character is not as simple as it seems, and that the sensory effect is not caused by one or two chemical compounds acting by themselves, but is rather the result of the interaction of these compounds. An interesting finding is the fact that the Elastoplast™ descriptor is not only affected by 4-ethylphenol or 4-ethylphenol and 4-ethylguaiacol, but also by 4-ethylcatechol and isovaleric acid. It is therefore

recommended that future studies on Brett character should focus on all four these compounds, and not only on 4-ethylphenol and 4-ethylguaiacol. This is of particular importance in the South African wine industry, as Pinotage contains excessive levels of the precursors of 4-ethylcatechol (de Villiers *et al.*, 2005), and is therefore more susceptible to elevated levels of this compound.

Although it is tempting to extrapolate these exciting findings into the real world situation, it still only scratches the surface of the situation that is sensory Brett character. Sensory effects in wine are affected by a variety of factors such as cultivar, wine style, alcohol content and the degree of inherent fruitiness the wine possesses (Le Berre *et al.*, 2007; Escudero *et al.*, 2007). This has also been found to be the case with Brett character (Norris, 2004; Suarez *et al.*, 2007). As this study highlighted the interactions between these compounds, the next step is to further investigate these interactions in a variety on wines, as well as in a variety of different combinations. Should this be done, Brett character can be modelled more successfully, and chemical analysis could become a better tool for the prediction of sensory Brett character.

6 REFERENCES

Atanasova, B., Thomas-Danguim, T., Langious, D., Nickluas, S., Chabanet, C., & Etievant, P. (2005a) Perception of wine fruity and woody notes: influence of peri-threshold odorants. *Food Quality and Preference*. **16**, 504 - 510.

Atanasova, B., Thomas-Danguin, T., Chabanet, C., Langious, D., Nickluas, S., & Etievant, P. (2005b) Perceptual interactions in odour mixtures: odour quality in binary mixtures of woody and fruity wine odorants. *Chemical Senses*. **30**, 209 - 217.

Atanasova, B., Thomas-Danguin, T., Langious, D., Nickluas, S., & Etievant, P. (2004) Perceptual interactions between fruity and woody notes of wine. *Flavour and Fragrance Journal*. **19**, 476 - 482.

Bro, R. (1997) PARAFAC. Tutorial and applications. *Chemometrics and Intelligent Laboratory Systems*. **38**, 149 - 171.

Bro, R., Qannari, E. M., Kiers, H. A. L., Næs, T. & Frøst, M. B. (2008) Multi-way models for sensory profiling data. *Journal of Chemometrics*. **22**, 36 - 45.

Brodin, M., Moeller, P. & Olsson, M. (2007) Are we mixing odorants or odors? (Abstract) *Chemical Senses*. **32**, A19.

Brossard, C., Rousseau, F. & Dumont, J. (2007) Perceptual interactions between characteristic notes smelled above aqueous solutions of odorant mixtures. *Chemical Senses*. **32**, 319 - 327.

Charters, S. & Pettigrew, S. (2007) The dimensions of wine quality. *Food Quality and Preference*. **15**, 997 - 1009.

Chatonnet, P., Dubourdieu, D., Boidron, J. & Pons, M. (1992) The origin of ethylphenols in wines. *Journal of the Science of Food and Agriculture*. **60**, 165 - 175.

Cheng, H. & Friis, A. (2008) *Expansion of the whole wheat flour extrusion*. Paper presented at 5th International Conference on Simulation and Modelling in the Food and Bio-Industry - FoodSim 2008, Dublin, Ireland, June 26-28, 2008. .

Cliff, M. A. & King, M. C. (2009) Influence of serving temperature and wine type on perception of ethyl acetate and 4-ethylphenol in wine. *Journal of Wine Research*. **20**, 45 - 52.

Cocchi, M., Bro, R., Duranye, C., Manzini, D., Marchetti, A., Saccani, F., Singhinolfi, S. & Ulrici, A. (2006) Analysis of sensory data of Aceto Balsamica Tradizionale di Modena (ABTM) of different ageing by application of PARAFAC models. *Food Quality and Preference*. **17**, 419 - 428.

Curtin, C. Bramley, B. Cowey, G. Holdstock, M. Kennedy, E. Lattey, K. Coulter, A. Henschke, P. Francis, L. Godden, P. Sensory perceptions of 'Brett' and relationship to consumer preference. Blair, R.J.; Williams, P.J.; Pretorius, I.S. Proceedings of the thirteenth Australian wine industry technical conference, 29 July-2 August 2007, Adelaide, SA. : 207-211; 2008.

De Villiers, A., Majek, P., Lynen, F., Crouch, A., Lauer, H. & Sandra, P. (2005) Classification of South African red and white wines according to grape variety based on the non-coloured phenolic content. *European Food Research and Technology*. **221**, 520 - 528.

Esbensen, K. H. (2002). Introduction to experimental design. In: *Multivariate Data Analysis*, 5th ed. Pp 361 - 446. Osla, Norway: CAMO Process AS

Escudero, A., Campo, E., Fariña, L., Cacho, J. & Ferreira, V. (2007) Analytical characterization of the aroma of five premium red wines. Insights into the role of odor families as the concept of Fruitiness of Wines. *Journal of Agricultural and Food Chemistry*. **55**, 4501 - 4510.

Eteiévant, P. X. (1981) Volatile phenol determination in wine. *Journal of Agricultural and Food Chemistry*. **29**, 65 - 67.

Eteiévant, P. X., Issanchou, S. N., Marie, S., Ducruet, V. & Flanzy, C. (1989) Sensory impact of volatile phenols on red wine aroma: influence of carbonic maceration time and storage. *Sciences des aliments*. **9**, 19 - 31.

Fariña, L., Boido, E., Carrau, F. & Dellacassa, E. (2007) Determination of volatile phenols in red wines by dispersive liquid-liquid microextraction and gas chromatography-mass spectrometry detection. *Journal of Chromatography A*. **1157**, 46 - 50.

Francis, I. L. & Newton, J. L (2005) Determining wine aroma from compositional data. In: *AWRI – Advances in wine science* (edited by R. J. Blair, M. E. Francis & I. S. Pretorius A. L.), Pp 201 - 212. Glen Osmond, Australia: Australian Wine Research Institute.

Fugelsang, K. C. & Zoeklein, B. W. (2003) Population dynamics and effects of *Brettanomyces bruxellensis s*trains on Pinot noir (Vitis vinifera L.) wines. *American Journal of Enology and Viticulture*. **54**, 294 - 300.

Glass, G.V., Peckham, P.D. & Sanders, J.R. (1972). Consequences of failure to meet assumptions underlying the fixed effects analyses of variance and covariance. *Review of Educational Research*, **42**, 237-288.

Grosh, W. (2001) Evaluation of the key odorants of foods by dilution experiments, aroma models and omission. *Chemical Senses*. **26**, 533 - 545.

Hesford, F. & Schneider, K. (2004) Discovery of a third ethylphenol contributing to *Brettanomyces* taint. *Obst- und Weinbau*. **140**, 11-13.

Hesford, F., Schneider, K., Porret, N. & Gafner, J. (2004) Identification and analysis of 4-ethylcatechol in wines tainted by *Brettanomyces* off-flavor (Abstract). *American Journal of Enology and Viticulture*. **55**, 304A.

Hughson, A. L. & Boakes, R. A. (2002) The knowing nose: the role of knowledge in wine expertise. *Food Quality and Preference*. **13**, 463 - 472.

Jinks, A. & Laing, D. G. (1999) Temporal processing reveals a mechanism for limiting the capacity of humans to analyse odor mixtures. *Cognitive Brain Research*. **8**, 311 - 324.

Laing, D. G. (1988) Relationship between the differential adsorption of odorants by the olfactory mucus and their perception in mixtures. *Chemical Senses*. **13**, 463 - 471.

Larcher, R., Nicolini, G., Bertoldi, D. & Nardin, T. (2008) Determination of 4-ethylcatechol in wine by high-performance liquid chromatography-coulometric electrochemical array detection. *Analytica Chimica Acta*. **609**, 235 - 240.

Laska, M. & Hudson, R. (1991) A comparison of the detection thresholds of odour mixtures and their components. *Chemical Senses*. **16**, 651 - 662.

Lattey, K. A., Bramley, B. R., Francis, I. L., Herderich, M. J. & Pretorius, S. (2007) Wine quality and consumer preferences: understanding consumer needs. *Australian and New Zealand Wine Industry Journal*. **22**, 31 - 39.

Lawless, H. T. & Heymann, H. (1998). *Sensory evaluation of food, principles and practice*. New York, USA: Chapman and Hall.

Lawless, H. T. (1999) Descriptive analysis of complex odors: reality, model or illusion. *Food Quality and Preference*. **10**, 325 - 332.

Le Berre, E., Atanasova, B., Langloi, Etiéviant, P. & Thamas-Danguin, T. (2007) Impact of ethanol on the perception of wine odorant mixtures. *Food Quality and Preference*. **18**, 901 - 908.

Le Berre, E., Béno, N., Ishii, A., Chabanet, C., Etiéviant, P. & Thomas-Danguin, T. (2008b) Just noticable differences in component concentrations modify the odor quality of a blending mixture. *Chemical Senses*. **33**, 489 - 395.

Le Berre, E., Thomas-Danguin, T., Béno, N., Coureaud, G., Etiéviant, P. & Prescott, J. (2008a) Perceptual processing strategy and exposure influence the perception of odor mixtures. *Chemical Senses*. **33**, 193 - 199.

Licker, J. L, Acree, T. E. & Henick-Kling, T. (1999) What is "Brett" (*Brettanomyces*) flavour? A preliminary investigation. In: *Chemistry of Wine Flavour. ACS Symposium Series* (edited by A. L. Waterhouse & S. E. Ebeler), Pp 96 - 115. Washington DC: American Chemical Society.

Livermore, A. & Laing, D. G. (1996) Influence of training and experience on the perception of multicomponent odor mixtures. *Journal of Experimental Psychology*. **22**, 267 - 277.

Masino, F., Foca, G., Ulrici, A., Arru, L. & Antonelli, A. (2008) A chemometric study of pesto sauce appearance and of its relation to pigment concentration. *Journal of the Science of Food and Agriculture*. **88**, 1335 - 1343.

Meilgaard, M. C., Civille, G. V. & Carr, B. T. (2007) *Sensory Evaluation Techniques*. Baco Raton, USA: CRC Press.

Mirhosseini, H & Tan, C. P. (2009) Response surface methodology and multivariate analysis of equilibrium headspace concentration of orange beverage emulsion as function of emulsion composition and structure. *Food Chemistry*. **115**, 324-333.

Mirhosseini, H., Tan, C. P., Hamid, N. S. A. & Yusof, S. (2008) Effect of Arabic gum, xanthum gum and orange oil on flavor release from diluted orange beverage emulsion. *Food Chemistry*. **107**, 1161-1172.

Noble, A.C., Arnold, R.A., Buechsenstein, J., Leach, E. J., Schmidt, J. O. & Stern, P. M. (1987) Modification of a standardized system of wine aroma terminology. *American Journal of Enology and Viticulture*. **38**, 143 - 146.

Norris, L. (2004) Unraveling the mystery of *Brettanomyces* flavour (Abstract). *American Journal of Enology and Viticulture*. **55**, 304A.

Oelofse, A., Lonvaud-Funel, A. & du Toit, M. (2009) Molecular identification of *Brettanomyces bruxellensis* strains isolated from red wines and volatile phenol production. *Food Microbiology*. **26**, 377 - 385.

Pravoda, V., Boucon, C., de Jong, S., Walczak, B. & Massart, D. L. (2002) Three-way principal component analysis applied to food analysis: an example*. Analytica Chimica Acta*. **462**, 133 - 148.

Prescott, J., Norris, L., Kunst, M. & Kim, S. (2005) Estimating a "consumer rejection threshold" for cork taint in white wine. *Food Quality and Preference*. **16**, 345 - 349.

Risvik, E. (2008) *From Simplicity to Complexity in Sensory Science*. Paper presented SAAFost Sensory Symposium, Cape Town, South Africa, August 13, 2008.

Romano, A., Perello, M. C., Lonvaud-Funel, A., Silcard, G. & de Revel, G. (2009) Sensory and analytical re-evaluation of “Brett character”. *Food Chemistry*. **114**, 15 - 19.

Saurez, R., Saurez-Lepe, J. A., Morata, A. & Calderon, F. (2007) The production of ethylphenols in wine by yeasts of the genera *Brettanomyces* and *Dekkera*: A review. *Food Chemistry*. **102**, 10-21.

Shapiro, S. S. & Wilk, M. B. (1965). An analysis of variance test for normality (complete samples). *Biometrika*, **52**, 591-611.

Tenenhaus, M., Pagès J., Ambroisine, L. & Guinot, C. (2004) PLS methodology to study relationships between hedonic judgements and product characteristics. *Food Quality and Preference*. **16**, 315 - 325.

Ugarte, P., Agosin, E., Bordeu, E. & Villalobos, J. I. (2005) Reduction of 4-ethylphenol and 4-ethylguaiacol concentration in red wines using reverse osmosis and adsorption. *American Journal of Enology and Viticulture*. **56**, 30 – 36.

Venkateshwarlu, G., Let, M. B., Meyer, A. S. & Jacobs, C. (2004) Modeling the sensory impact of defined combinations of volatile lipid oxidation products on fishy and metallic off-flavours. *Journal of Agricultural and Food Chemistry*. **52**, 1635 - 1641.

Wirz, D. O., Heymann, H. & Bisson, L. F. (2004) Descriptive analysis of *Brettanomyces*-infected Cabernet Sauvignon wines. *American Journal of Enology and Viticulture*. **55**, 303A.

Wise, B. M., Gallagher, N. B., Bro, R., Shaver, J. M., Windig, W., and Koch, R. S. PLS Toolbox. [Version 4.3]. 2009. Eigenvector Research Inc., Manson, WA.

Chapter 6: Explorative investigation into the incidence of eight *Brettanomyces*-related spoilage compounds in a selection of South African red wines

1 INTRODUCTION

The chemical analysis of 4-ethylphenol and 4-ethylguaiacol is commonly used for the diagnosis of wines spoiled by *Brettanomyces*. However, poor correlations have been found between the levels of these compounds and sensory attributes associated with this yeast (Fugelsang & Zoecklein, 2003; Romano *et al*, 2009). Recent studies have tended to not only include 4-ethylphenol and 4-ethylguaiacol, but several other compounds as well. These include predominantly 4-ethylcatechol (Curtin *et al*., 2008), as well as isovaleric acid (Romano *et al*., 2009).

As 4-ethylcatechol was only linked to the spoilage of red wine in 2004, very few studies have been performed investigating this compound. This is partly due to the fact that the chemical characteristics of 4-ethylcatechol necessitates the use of a different method of chemical analysis than the "classical" methods used for the other volatile phenols. 4-ethylcatechol may be analysed by gas chromatography (GC) preceded by a derivitisation step (Hesford & Schneider, 2004; Carillo & Tena, 2007) or by means of high performance liquid chromatography (HPLC) (Larcher *et al*., 2008)

The aim of this study was to determine the concentrations of eight Brett-related spoilage compounds (4-ethylphenol, 4-ethylguaiacol, 4-ethylcatechol, 4-vinylphenol, 4-vinylguaiacol, isovaleric acid, isobutyric acid and acetic acid) in a selection of South African red wines. The sample set was on purpose selected to contain a large proportion of wines potentially spoiled by *Brettanomyces*, especially with the aim to investigate the occurrence of 4-ethylcatechol.

2 MATERIALS AND METHODS

2.1 Samples

Thirty-four red wine samples were sourced from the South African wine industry. Of these thirty-two samples, twelve were obtained from a wine analysis facility, and were considered to be spoiled with *Brettanomyces*. Twenty wines were directly obtained from South African wine cellars, of which ten were already on the market. The remaining two South African wines were the wines used for the sensory analysis component of this study.

2.2 Chemical analyses

Analysis of 4-ethylphenol, 4-ethylguaiacol, 4-vinylphenol, 4-vinylguaiacol, isovaleric acid, isobutyric acid and acetic acid was performed using GC-MS. Extraction was performed using 2

mL diethyl ether in 10 mL of wine, using 2,3- dimethyl phenol (50 µg/L) (Sigma Aldrich, Germany) as internal standard The samples were subsequently sonicated for 30 minutes (shaken at 5 minute intervals) and then dried over sodium sulphate. The organic phase of each extraction was collected, and 2 µL of the extract was injected into an Agilent 5890 GC-MS. The column employed was a DB-FFAP (60m x 320.0 µm x 0.5 µm column) and the carrier gas was helium. The injector (split/splitless) was heated to 260 ºC with a splitless time of 1 minute and a split flow of 1.2 mL/min. The oven temperature was increased after injection from 40 ºC at a rate of 20 ºC/min up to 150 ºC and then at 5 ºC/min up to 240 ºC, where it was held for 8 minutes. MS quantisation was performed in selected ion monitoring (SIM) mode. The relevant ions are shown in Table 6.1.

Table 6.1. Ions used for MS analysis in SIM mode.

Compound	Ions
4-ethylphenol	107, 122
4-ethylguaiacol	137, 152
4-vinylphenol	91, 120
4-vinylguaiacol	135, 150
Isovaleric acid	60, 69, 87
Isobutyric acid	73, 88, 55
Acetic acid	46, 60, 61
2,3-dimethylphenol	107, 122

The analysis for 4-ethylcatechol was performed using HPLC with tandem mass spectrometric (MS/MS) detection. This method was chosen as difficulties were experienced in developing a GC method suitable for analysis of this compound. A Waters Alliance 2695 liquid chromatograph (Waters Corporation, Milford, U. S. A.) equipped with a Waters API Quattro Micro tandem quadruple mass spectrometric detector was used for this analysis. Sample extracts were separated by reversed phase liquid chromatography utilizing an acetonitrile and water gradient and a C18 column (Waters Xbrige, 2.1 x 50 mm with guard) The gradient started at 5% acetonitrile, increased to 90% in 6 minutes, followed by a clean-up step consisting of 95% water for 4 minutes. The flow was maintained at 0.4 mL/min throughout. Negative electrospray ionization was performed using the following optimized parameters: cone voltage 15 V, capillary voltage, 3.5 kV, source and desolvation temperatures 100 ºC and 400 ºC respectively. Nitrogen was used as desolvation and cone gas at flow rates of 400 L/h and 50 L/h respectively. The mass spectrometer was operated in multiple reaction monitoring mode. Acquisition parameters are given in Table 6.2.

The calibration for this method was performed using standards done between 10 µg/L and 500 µg/L, as well as wine samples up to 500 µg/L. The R^2 for the calibration curve was 0.99.

Table 6.2. Parameters for aquisition of MRM data.

Parent ion (Da)	Collision Energy (eV)	Daughter ion (Da)
119.0	20	93.0
122.0	15	108.0
136.8	15	122.0
149.0	15	134.20
151.0	15	136.20

2.3 Statistical analysis

Principal component analysis (PCA) was performed on quantitative data for the analysed South African wines. Linear regression was performed to investigate the relationships between the compounds between different sets of compounds between the data. The mean, median, minimum and maximum values were also calculated for each compound. All these analyses were performed using the XLStat software (Version 2009.5.0.1 Addinsoft, SARL, Paris, France).

3 RESULTS AND DISCUSSION

3.1 Quantitative results

A summary of the overall results can be seen in Table 6.3, which reveals some interesting aspects of the dataset. Firstly, the maximum level of 4-ethylphenol found in the dataset was 16330 µg/L. This value is extremely high, as it exceeds the value mentioned in literature (Francis & Newton, 2005) by approximately four times. It is also four times as high as the value used in this study (Chapters 4 and 5). However, this wine was identified as being excessively spoiled. Another interesting aspect is the fact the maximum level of 4-ethylcatechol found was only 227.7 µg/L, in spite of the fact that some of the wines analysed were severely spoiled. This value is lower than the detection threshold found in this study (385 µg/L, see Chapter 3), as well as the maximum value of 1610 µg/L found by Larcher *et al*. (2008). However, the median, first quartile (25^{th} percentile) and third quartile (75^{th} percentile) showed good agreement with the latter study.

Figure 6.1 and Figure 6.2 show the results of PCA performed on the entire dataset. In these figures, all the compounds except for 4-ethylcatechol are highly correlated, and the variation in their levels explain most of the variation in the dataset (PC1 76%). It is interesting to note that loadings for 4-ethylphenol and 4-ethylguaiacol lie close together, as do the loadings for isobutyric and isovaleric acid. However, the loading value for 4-ethylcatechol does not

correlate with the other variables. From this it can be inferred that the levels of 4-ethylcatechol in this sample set are not correlated to the levels of 4-ethylphenol and 4-ethylguaiacol for the analysed samples. Furthermore, the six samples to the right of Figure 6.2 contain the highest levels of Brett spoilage compounds, and it can therefore be said that PC 1 explains Brett spoilage. 4-ethylcatechol explains most of the variation in PC 2. It can thus be concluded that the levels of 4-ethylcatechol in this sample set cannot be directly linked to Brett spoilage.

Table 6.3. Summary of results of chemical analyses of *Brettanomyces* derived compounds of 34 South African red wines.

	Mean	25th percentile	Median	75th percentile	95th percentile	Maximum
4-ethylphenol (µg/L)	1591	54	404	687	5368	16330
4-ethylguaiacol (µg/L)	146	11	39	71	419	1236
4-ethylcatechol (µg/L)	47	8	30	56	114	228
4-vinylphenol (µg/L)	6278	785	1379	3310	19063	58685
4-vinylguaiacol (µg/L)	283	45	70	178	1301	1871
Isovaleric acid (µg/L)	2780	485	838	2010	11349	18995
Isobutyric acid (µg/L)	759	133	217	478	3196	5103
Acetic acid (g/L)	2.80	0.44	0.83	1.59	11.82	14.98

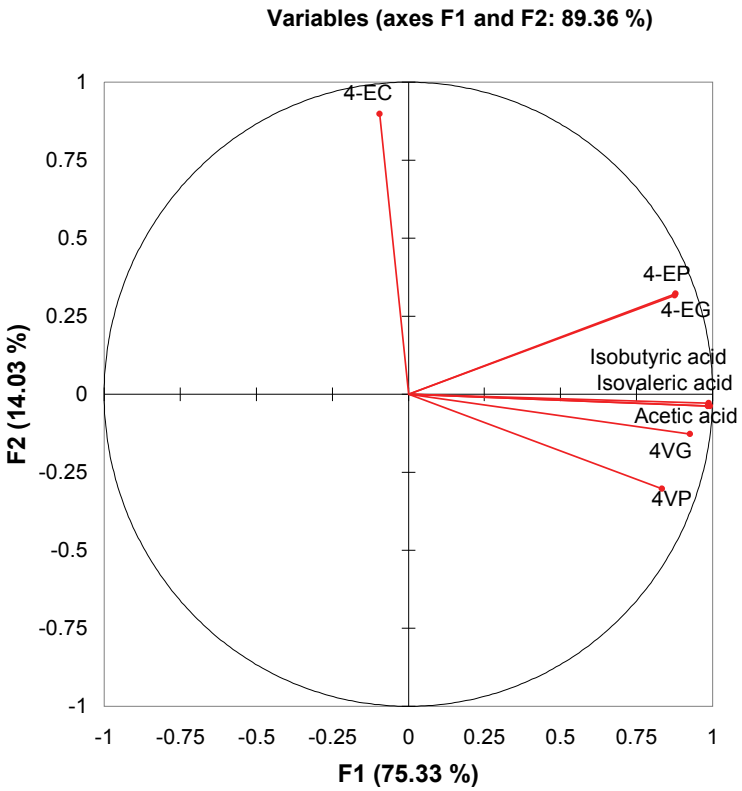


Figure 6.1. Principal component analysis loadings plot on for the chemical data obtained from 34 red wine samples. Compounds included are 4-ethylphenol (4-EP), 4-ethylguaiacol (4-EG) 4-ethylcatechol (4-EC), 4-vinylphenol (4-VP), 4-vinylguaiacol (4-VG), isovaleric acid, isobutyric acid and acetic acid.

A second principal component analysis was performed on a selected sample set (Figure 6.3). The samples in this set were selected by determining the total value for 4-ethylphenol and 4-ethylguaiacol in the sample. If this number fell between 426 µg/L (the diagnostic value determined by Chatonnet *et al*., 1992) and 5000 µg/L (the maximum combined concentration used in this study), the sample was included in the dataset. This was done to only include samples that were spoiled by *Brettanomyces*, but to exclude those that were severely spoiled. This allowed for a more detailed investigation into the smaller differences in chemical composition between the spoiled samples. A PCA biplot for this analysis is shown in Figure 6.3.

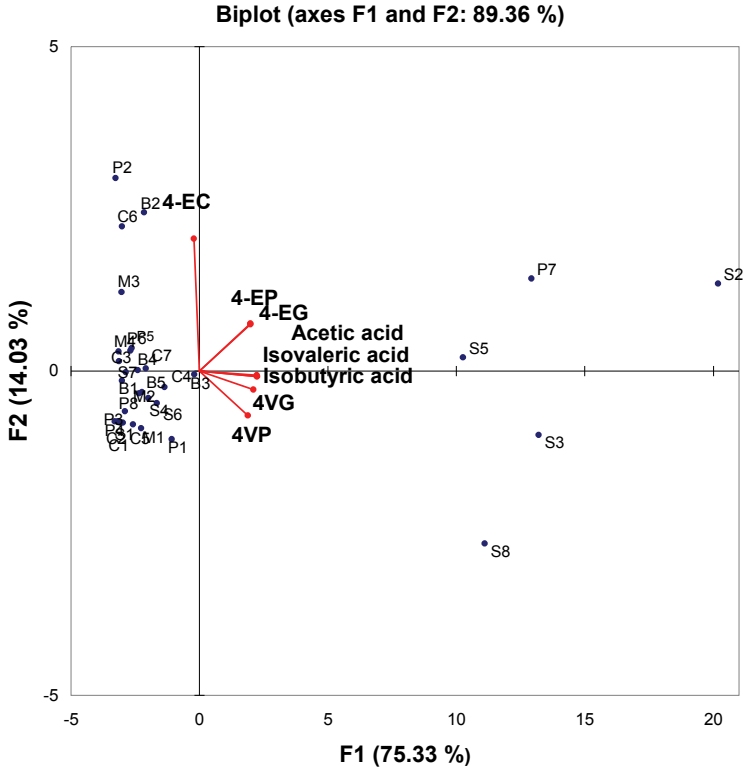


Figure 6.2. Principal component analysis biplot on chemical data obtained from 34 red wine samples. Sample names refer to the cultivar contained in the sample (B – Blend; C – Cabernet sauvignon; M – Merlot; P – Pinotage; S – Shiraz).

The results obtained do suggest that cultivar has an influence in the amount of 4-ethylcatechol present in a sample, as the three Pinotage samples in the biplot tend to associate with the loading for 4-ethylcatechol. This suggests that the hypothesis that this cultivar is more subject to higher levels of 4-ethylcatechol due to its higher levels of precursors may be valid.

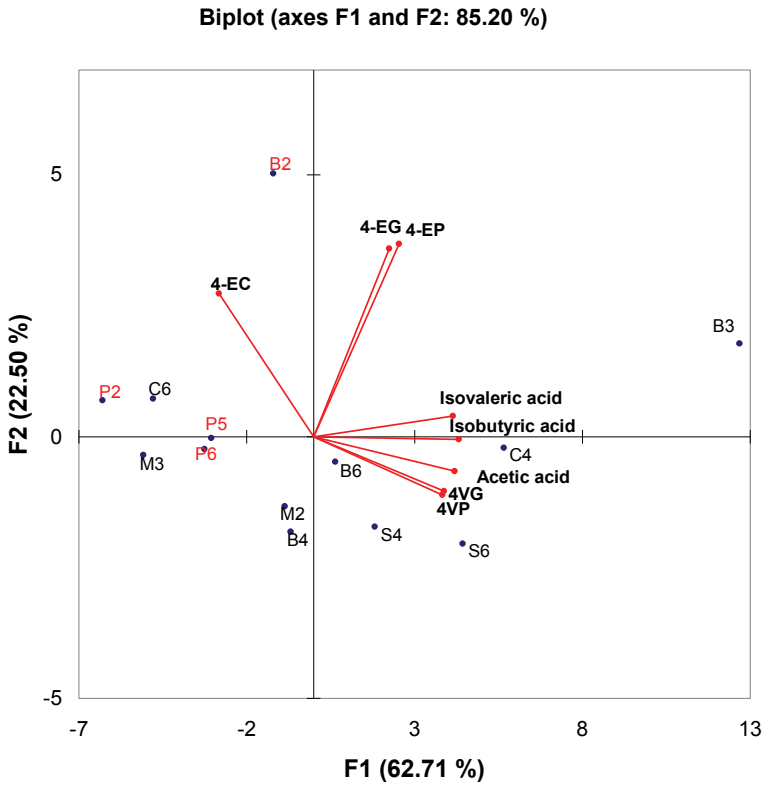


Figure 6.3. Principal component analysis of samples where the sum of 4-ethylphenol and 4-ethylguaiacol was between 426 µg/L and 5000 µg/L. Sample names refer to the cultivar contained in the sample (B – Blend; C – Cabernet sauvignon; M – Merlot; P – Pinotage; S – Shiraz). Pinotage samples, as well as Pinotage-based blends are highlighted in red.

3.2 Relationships between compound levels

Linear regression analysis was performed on sets of compounds shown in Table 6.3. The results are summarised in Table 6.4.

Table 6.4. Results of regression analysis of the quantitative data for all compounds.

Compounds	P for regression	R^2 value
4-EP and 4-EG	< 0.0001	0.966
4-EP and 4-EC	0.785	0.003
4-EP and isovaleric acid	< 0.0001	0.888
4-EP and isobutyric acid	< 0.0001	0.900
4-EG and 4-EC	0.816	0.002
4-EG and isovaleric acid	< 0.0001	0.854
4-EG and isobutyric acid	< 0.0001	0.874
Isovaleric acid and isobutyric acid	< 0.0001	0.996

The first interesting aspect of Table 6.4 is the fact that significant correlations were found between all the sets of variables except for the ones containing 4-ethylcatechol. This is

particularly unexpected since 4-ethylcatechol is produced by the same enzymatic pathway as 4-ethylphenol and 4-ethylguaiacol, and a relationship is therefore expected. However, Larcher *et al*. (2008) pointed out that cultivar has a significant effect on the production of 4-ethylcatechol, which might explain the results in Table 6.4.

Figure 6.4, Figure 6.5 and Figure 6.6 show the three most significant relationships listed in Table 6.4. As it can be seen, there is a strong linear relationship (R^2 = 0.97, Figure 6.4) between the level of 4-ethylphenol and 4-ethylguaiacol in the sample set. This is expected, as these two compounds are produced by the same enzymatic pathway. A similarly strong relationship (R^2 = 0.97 Figure 6.6) is exhibited between the levels of isovaleric acid and isobutyric acid. This is also likely that they are formed by the same biochemical pathway (Harwood & Canale-Parola, 1981). However, the most interesting aspect is depicted in Figure 6.5. This figure shows a strong correlation (R^2 = 0.88) between the level of 4-ethylphenol and isovaleric acid in the samples. This relationship could also be observed between other 4-ethylphenol and isobutyric acid, as well as 4-ethylguaiacol and both isovaleric and isobutyric acid (Table 6.4). This relationship is in agreement with the findings of Romano *et al*. (2009), and is further evidence that all four these compounds contribute to the sensory effect which is Brett character.

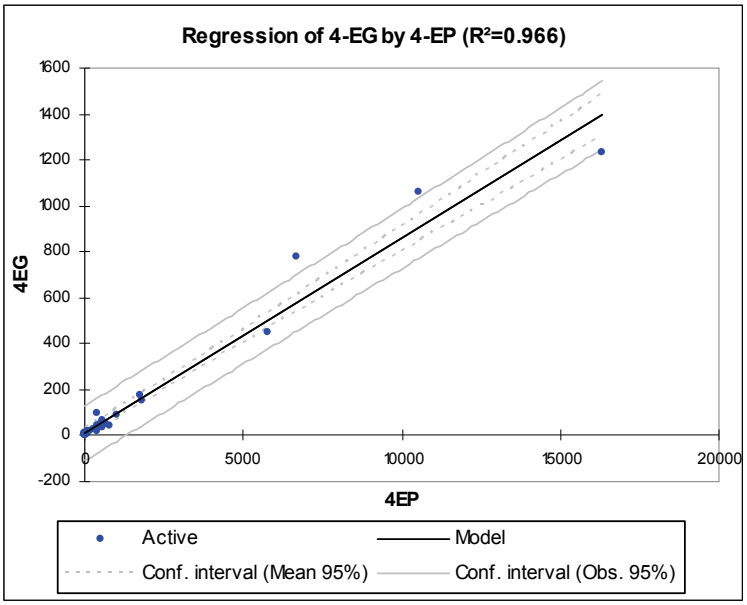


Figure 6.4. Relationship between 4-ethylphenol and 4-ethylguaiacol content of selected South African red wines. The curve has the following equation: 4-EG = 10.50+0.085*4-EP

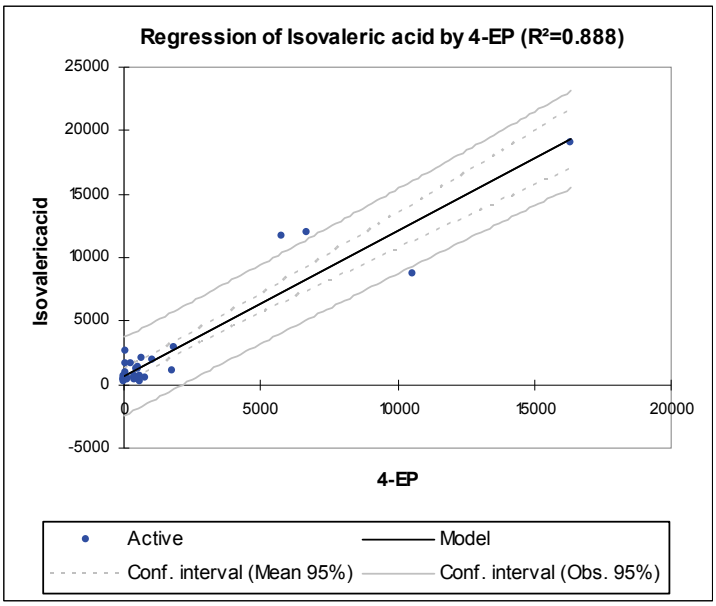


Figure 6.5. Relationship between 4-ethylphenol and isovaleric acid in selected South African red wines. The curve satisfies the following equation: Isovaleric acid = 618+1.14*4EP

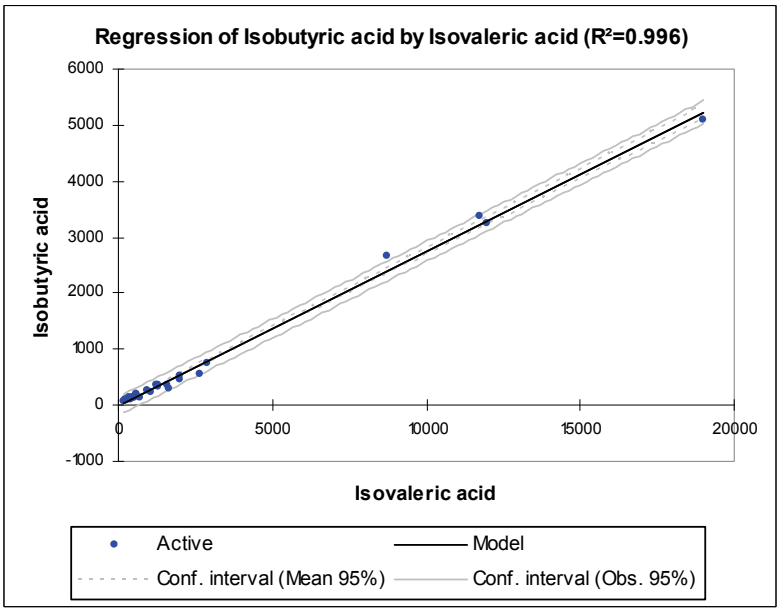


Figure 6.6. Relationship between isovaleric acid and isobutyric acid in selected South African red wines. The curve has the following equation: Isobutyric acid = -12.34 +0.26 * Isovaleric acid

There are two possible explanations for the poor correlations found between the levels of 4-ethylcatechol and the other two ethylphenols used in this study. The first, which has already been mentioned, is the effect of cultivar. It is likely that differences in the levels of precursors between the respective cultivars lead to different levels of the spoilage compounds. This would be in agreement with the findings of Hesford *et al*. (2004) and Larcher *et al*. (2008), who investigated these effects. However, in order to prove this hypothesis, a more comprehensive study containing more representative numbers of each cultivar needs to be undertaken. The

second possible cause of this phenomenon is microbiological. In their review on *Brettanomyces* in wine, Renouf *et al.* (2007) presents the theory that this yeast decarboxilates the hydrocinnamic acids in order to protect itself against their toxic effects. Should caffeic acid show a different (lower) toxicity towards *Brettanomyces* than *p*-coumaric and ferulic acid, it may result in a lower production of 4-ethylcatechol.

4 CONCLUSIONS

This study was the first to investigate the levels of 4-ethylcatechol found in selected South African red wines. It was found that the levels of 4-ethylcatechol are not related to the levels of 4-ethylguaiacol and 4-ethylphenol found, which is likely that this is due to the cultivar effect. However, strong correlations were found between the levels of 4-ethylphenol and both isovaleric and isobutyric acids. It can therefore be concluded that these carboxylic acids are formed by *Brettanomyces* alongside the ethylphenols. In order to investigate the effect of cultivar on the levels of 4-ethylcatechol produced by *Brettanomyces*, it is recommended that a larger study should be undertaken investigating a more representative sample set.

5 REFERENCES

Carrillo, J. D. & Tena, M. T. (2007) Determination of ethylphenols in wine by in situ derivitisation and headspace solid-phase microextraction-gas chromatography-mass spectrometry. *Analytical and Bioanalytical Chemistry*. **387**, 2547 - 2558.

Chatonnet, P., Dubourdieu, D., Boidron, J. & Pons, M. (1992) The origin of ethylphenols in wines. *Journal of the Science of Food and Agriculture*. **60**, 165 - 175.

Curtin, C. Bramley, B. Cowey, G. Holdstock, M. Kennedy, E. Lattey, K. Coulter, A. Henschke, P. Francis, L. Godden, P. Sensory perceptions of 'Brett' and relationship to consumer preference. Blair, R.J.; Williams, P.J.; Pretorius, I.S. Proceedings of the thirteenth Australian wine industry technical conference, 29 July-2 August 2007, Adelaide, SA. : 207-211; 2008.

Francis, I. L. & Newton, J. L (2005) Determining wine aroma from compositional data. In: *AWRI – Advances in wine science* (edited by R. J. Blair, M. E. Francis & I. S. Pretorius A. L.), Pp 201 - 212. Glen Osmond, Australia: Australian Wine Research Institute.

Fugelsang, K. C. & Zoeklein, B. W. (2003) Population dynamics and effects of *Brettanomyces bruxellensis* strains on Pinot noir (Vitis vinifera L.) wines. *American Journal of Enology and Viticulture*. **54**, 294 - 300.

Harwood C. S. & Canale-Parola, E. (1981) Adenosine-5'-Triphosphate-yielding pathways of branched-chain amino acid fermentation by a marine Spirochete. *Journal of Bacteriology*. **148**, 117 - 123.

Hesford, F. & Schneider, K. (2004) Discovery of a third ethylphenol contributing to *Brettanomyces* taint. *Obst- und Weinbau*. **140**, 11-13.

Hesford, F., Schneider, K., Porret, N. & Gafner, J. (2004) Identification and analysis of 4-ethyl catechol in wines tainted by *Brettanomyces* off-flavor. *American Journal of Enology and Viticulture*. **55**, 304A.

Larcher, R., Nicolini, G., Bertoldi, D. & Nardin, T. (2008) Determination of 4-ethylcatechol in wine by high-performance liquid chromatography-coulometric electrochemical array detection. *Analytica Chimica Acta*. **609**, 235 - 240.

Renouf, V., Louvaud-Funel, A. & Coulon, J. (2007) The origin of *Brettanomyces bruxellensis* in wines: A review. *Journal Internationale de Science de Vigne et Vin*. **41**, 161 - 173.

Romano, A., Perello, M. C., Lonvaud-Funel, A., Silcard, G. & de Revel, G. (2009) Sensory and analytical re-evaluation of "Brett character". *Food Chemistry*. **114**, 15 – 19

.

Chapter 7: General discussion and conclusions

This study aimed to investigate in detail the sensory effects of several Brett-related spoilage compounds by first identifying their detection thresholds, profiling them separately and profiling them in combination. All sensory experiments in this study were performed on Pinotage red wine spiked with the relevant compounds.

When establishing the detection thresholds of eight compounds (4-ethylphenol, 4-ethylguaiacol, 4-ethylcatechol, isovaleric acid, isobutyric acid, 4-vinylphenol, 4-vinylguaiacol and acetic acid) it was found that large variation existed in the ability of judges to detect these different compounds. This is in agreement with the findings of Curtin *et al*. (2008) regarding Brett-related compounds. It was also found that using the median for these determinations was a better way of dealing with this type of data than traditional mean-based methods, as the median gave a better indication of the distribution of detection throughout the population.

The differences between the detection thresholds found in this study and those in literature could generally be explained by the difference in medium, which underlines the importance of the determination of detection thresholds in the relevant medium before undertaking a major sensory study. Two major discrepancies were however found considering the literature values traditionally quoted. These were 4-ethylphenol (195 vs 605 ug/L found by Chatonnet *et al*. (1992)) and 4-ethylcatechol (385 vs 60 ug/L found by Hesford and Schneider (2004)). However, both values found in this study fell closer to values more recently determined, namely 368 µg/L for 4-ethylphenol (Curtin *et al*., 2008) and 100 – 400 µg/L for 4-ethylcatechol (Larcher *et al*., 2008).

Only four compounds – 4-ethylphenol, 4-ethylguaiacol, 4-ethylcatechol and isovaleric acid – were investigated in the remainder of the study. This choice was made as these four compounds are most commonly linked to Brett character. Limiting the number of compounds to be profiled in combination also produced a sample-set that could be easily managed by the sensory panel.

Profiling 4-ethylphenol, 4-ethylguaiacol, 4-ethylcatechol and isovaleric acid produced predictable results. An increase of 4-ethylphenol concentration caused an increase in Elastoplast™ and leather-like attributes and an increase in 4-ethylguaiacol concentration caused an increase in medicinal and smoky attributes. An increase in 4-ethylcatechol concentration caused an increase in the savoury attribute, and an increase in isovaleric acid concentration cause an increase in the pungent attribute. All four these compounds were found to suppress berry-like character in the wines, and to produce a sick-sweet, confected aroma. This aroma was concluded to be the direct result of the suppression of the natural berry-like character in wines.

However, when these compounds were profiled in combination, their combined effect was significantly different than their effect when present individually, and several second- and

third-order interactions were found. The most notable of these were the third-order interactions of 4-ethylphenol*4-ethylguaiacol*4-ethylcatechol and 4-ethylphenol*4-ethylguaiacol*isovaleric acid. It was found that both 4-ethylcatechol and isovaleric acid enhanced the Elastoplast™ attribute when 4-ethylguaiacol was present at its detection threshold. 4-ethylguaiacol was also found to enhance the Elastoplast™ effect when 4-ethylcatechol and isovaleric acid were above their detection thresholds. Furthermore, 4-ethylphenol, 4-ethylguaiacol and 4-ethylcatechol had different effects on the overall aroma of the wine below detection threshold than when above detection threshold. This is in line with the findings of Anatosova *et al.* (2004). We further found that 4-ethylphenol enhanced the pungent descriptor that is associated with isovaleric acid. These three interactions could be partially responsible for current unanswered questions and the controversy questions surrounding sensory Brett character, and therefore require further investigation.

Consumer analysis of wines with Brett character was largely inconclusive. It was found that the sample with the highest level of 4-ethylphenol was least liked by consumers, while the "control" sample was liked less by consumers than some of the samples with a degree of Brett character. It can therefore be concluded that Brett character can in some cases increase consumer liking of wine.

Although not the first study conducted on Brett character, this study is unique because it systematically investigated four Brett-spoilage compounds, whereas previous studies either omitted 4-ethylcatechol (Romano *et al*., 2009) or isovaleric acid (Curtin *et al*., 2008). However, due to the interactions found in this study, it can be concluded that all four compounds have a significant effect on Brett character. It is also likely that not only these four compounds interact in terms of Brett character, and that other compounds are also strongly involved. It is therefore recommended that future studies should include at least all four these compounds. This is of particular importance in the South African wine industry, as Pinotage, a uniquely South African cultivar, contains high levels of the precursors of 4-ethylcatechol (De Villiers *et al*., 2005) and could therefore be more susceptible to spoilage by this compound.

This study was the first performed in South Africa to include the chemical analysis of 4-ethylcatechol in red wine. Although a strong correlation was found between the levels of 4-ethylphenol and 4-ethylguaiacol (and isovaleric acid) present in samples, no correlation was found between the levels of these compounds and 4-ethylcatechol. The data suggests that cultivar or strain of *Brettanomyces* may play a role in this lack of correlation, which is in agreement with the results of Larcher *et al.* (2008). The next task would be to undertake a large-scale investigation into the prevalence of 4-ethylcatechol in South African red wines, especially Pinotage.

Although the present extensive investigation of the sensory effects of Brett character has shed some light on the complexity of the phenomenon, several important unanswered questions remain. While it has been known for many years that Brett character is complex, we now have a

better understanding regarding the reason for this complexity. This study thus paved the way for future research of the sensory effect of Brett infection in wine.

It is recommended that follow-up studies should be done on the same subject matter. This should take the form of a sensory investigation of the effects of these four compounds in the form of a factorial design (all combinations of all compounds to be tested). However, such a study would be limited in its concentration range, as the number of levels to be tested increases the number of samples exponentially. For instance, if only 3 levels were to be investigated, 81 samples would have to be studied, which is already a vast number for descriptive sensory analysis.

It is generally suggested that all future studies – whether sensory, chemical or microbiological – regarding Brett character should include analysis of 4-ethylcatechol and isovaleric acid. It has been shown in this study that these compounds do interact with the other spoilage compounds in terms of sensory effects. Any studies failing to include these compounds therefore potentially lack important information.

REFERENCES

Chatonnet, P., Dubourdieu, D., Boidron, J. & Pons, M. (1992) The origin of ethylphenols in wines. *Journal of the Science of Food and Agriculture*. **60**, 165 - 175.

Curtin, C. Bramley, B. Cowey, G. Holdstock, M. Kennedy, E. Lattey, K. Coulter, A. Henschke, P. Francis, L. Godden, P. Sensory perceptions of 'Brett' and relationship to consumer preference. Blair, R.J.; Williams, P.J.; Pretorius, I.S. *Proceedings of the thirteenth Australian wine industry technical conference,* 29 July-2 August 2007, Adelaide, SA. : 207-211; 2008.

De Villiers, A., Majek, P., Lynen, F., Crouch, A., Lauer, H. & Sandra, P. (2005) Classification of South African red and white wines according to grape variety based on the non-coloured phenolic content. *European Food Research and Technology*. **221**, 520 - 528.

Hesford, F. & Schneider, K. (2004) Discovery of a third ethylphenol contributing to *Brettanomyces* taint. *Obst- und Weinbau*. **140**, 11-13.

Larcher, R., Nicolini, G., Bertoldi, D. & Nardin, T. (2008) Determination of 4-ethylcatechol in wine by high-performance liquid chromatography-coulometric electrochemical array detection. *Analytica Chimica Acta*. **609**, 235 - 240.

Romano, A., Perello, M. C., Lonvaud-Funel, A., Silcard, G. & de Revel, G. (2009) Sensory and analytical re-evaluation of “Brett character”. *Food Chemistry*. **114**, 15 - 19.